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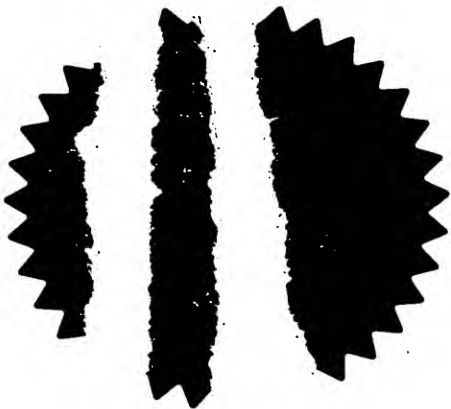
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Dated

1 September 2003







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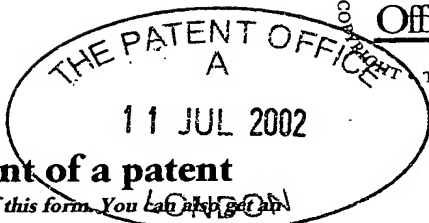
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HEALTH PROTECTION AGENCY,  
Porton Down,  
SALISBURY,  
Wiltshire,  
SP4 0JG,  
United Kingdom

Incorporated in the United Kingdom,

[ADP No. 08638108001]





1/77  
12JUL02 E732756-1 002000  
P01/7700 0.00-0216146.1

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2. Patent application number

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0216146.1

11 JUL 2002

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Microbiological Research A City  
CAMR  
Porton Down, Salisbury  
Wiltshire  
SP4 0JF

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

GB

62726229001

SECTION 30 (1977 ACT) APPLICATION FILED

4. Title of the invention

Degradation and detection of TSE infectivity

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

MATHYS AND SQUIRE  
100 Gray's Inn Road  
London WC1X 8AL  
United Kingdom

Patents ADP number (if you know it)

1081001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

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Priority application number  
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## DEGRADATION AND DETECTION OF TSE INFECTIVITY

5 The invention is in the field of methods and compositions for the sterilisation of materials and apparatus that may have been contaminated with infectious agents and for detection of those agents. In particular, the invention relates to methods for the inactivation and detection of transmissible spongiform encephalopathy (TSE) agents and provides compositions for degrading and detecting TSE located on or within infected materials.

10

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurological diseases that include Creutzfeld-Jacob disease (CJD) and Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle and Scrapie in sheep. TSEs are characterised by the conversion of a normal host protein into a pathogenic protein within the brain tissue of an infected animal. The pathogenic form of protein is often referred to as a prion and is highly resistant to physical and chemical degradation. The prion is believed to be the transmissible agent through which the TSE disease is passed on between animals.

15

20 There has been considerable public alarm in recent years over the risks associated with consumption of meat products, and especially beef, potentially infected with BSE, the bovine form of TSE. Much of this concern is associated with the belief that the BSE prion when eaten by a human may in some cases cause the incurable human form of the disease, referred to as variant CJD (vCJD). Rigorous practices have been adopted in the agricultural and meat rendering industries to reduce the risk of cross contamination between BSE infected carcasses and meat that is destined for human consumption or other animal derived products such as tallow. However, BSE infected animals can still be unintentionally processed in abattoirs, especially if the animal is in the early stages of the disease and therefore undetected as an infected TSE host. There is also a considerable risk in disposal of known BSE infected material, particularly if the equipment used in the disposal operation is then reused in normal rendering practices without adequate sterilisation.

25

30

Sterilisation of instruments and equipment following potential exposure to TSE infected tissue is of primary importance. In particular, surgical equipment such as scalpels, forceps and endoscopes should be thoroughly sterilised before use on patients to avoid disease transmission.

35

It has been reported that the CJD infectious agent was accidentally transferred on surgical electrodes, inserted into the brain of a human patient with CJD, to two other previously uninfected patients (Bernouli et al (1977) *The Lancet* i: pp478-479). The electrodes concerned were sterilised with ethanol and formaldehyde vapour between  
5 each procedure, conditions previously thought to be sufficient to eliminate virtually all infectious agents, and yet the CJD infectious agent was able to withstand such harsh conditions and infect the recipient patients' brain tissue.

TSE transmission is typically observed in cases where infected material is  
10 transferred between animals or implanted into an animal. As described previously, incomplete or inadequate sterilisation of surgical instruments can lead to such transfer of infected material between patients. Even the most rigorous chemical cleaning and steam sterilisation procedures can fail to remove blood and tissue from surgical instruments, especially in the jaws or joints of forceps and clamps  
15 (Laurenson (1999) *The Lancet*, 20 Nov). Thus, the risk of unintentional TSE transfer can be unnecessarily high.

TSE agents, or prions, are known to be highly resistant to denaturation and degradation, more so than would normally be expected for a protein. Taylor (J. Hosp.  
20 Infect. (1999) 43 supplement, pp S69-76) reviews a number of methods for inactivating prion proteins.

Chemical methods for inactivating TSE prion proteins include treatment of infected material with sodium hydroxide or sodium hypochlorite solutions (Taylor et al. (1994)  
25 Arch. Virol. 139, pp313-326), although infectivity of the prion is shown to survive exposure to 2M sodium hydroxide for up to two hours.

Alternative methods for inactivating TSE prions include autoclaving, but again BSE and scrapie agent has been shown to survive treatment at 134-138°C for 18 minutes  
30 (Taylor et al. *ibid*). Thus, a combined chemical/heating approach has been proposed in which infected materials are exposed to 1M sodium hydroxide followed by autoclaving at 121°C for between 30 and 60 minutes (Taylor, J. Hosp. Infect. (1999) 43 supplement, pp S69-76). This combined method has shown that inactivation of TSE infectious agents can be achieved, albeit under very harsh conditions.

35 However, many materials, such as plastics, polymers and non-protein animal derivatives, cannot be exposed to such extreme conditions without themselves being

destroyed. The chemical and physical processes described above are only really suitable for sterilising metal instruments and surgical tools that are not too large in size and which can fit inside a standard autoclave. More delicate instruments such as endoscopes cannot be exposed to extreme conditions of high temperature without the risk of permanent damage to their internal components.

Further, chemical processes typically involve the use of caustic and/or chaotropic agents which are hazardous to handle and dispose of. It would, therefore, be desirable to provide a method for inactivating agents such as TSE without the need to use large amounts of hazardous substances and which method could be scaled up for use on larger objects and areas as well as on smaller objects.

Taylor (Vet. J. (2000) 159 pp10-17) describes tests using proteolytic enzymes to deactivate prion proteins. Proteases such as trypsin have little effect in non-denaturing conditions (Taylor (2000) p. 14) but other proteases such as proteinase K may have an effect on TSE infectivity following prolonged digestion times. However, the majority of current TSE inactivation methods are aimed towards chemical and physical degradation procedures.

It is an object of the invention, therefore, to provide methods and means for effectively inactivating TSE infectious agents under conditions that can be readily applied to a variety of locations and situations. It is a further object of the invention to reduce the need for extreme conditions of very high temperature and harsh chemical denaturants in order to inactivate TSE agents located on or within TSE infected materials.

A first aspect of the invention provides a method for inactivating a TSE agent comprising exposing the TSE agent to a thermostable proteolytic enzyme.

The methods and compositions of the invention are suitable for the inactivation of TSE agent in apparatus and materials infected or suspected as being infected with TSE agent. In addition, the methods and compositions of the invention are suitably used in a prophylactic or precautionary mode, where definite knowledge of infection is uncertain. For example, the method of the invention can be easily incorporated into the standard sterilisation protocols used for preparation of surgical apparatus prior to use in surgical procedures.

The methods and compositions of the present invention are also suitably used for the inactivation of TSE agents in potentially contaminated clinical waste and culled animal material. At present, this waste material is incinerated at 1000°C, which requires specialised facilities and is expensive. It is an advantage of the present invention that a TSE inactivation procedure can occur at temperatures and in conditions which do not require highly specialised facilities and that the prospects of complete inactivation of the TSE agent are comparable to the more energy intensive and expensive incineration procedures.

The term transmissible spongiform encephalopathy (TSE) agent is intended to encompass all neurological diseases that are apparently transmitted via a pathogenic prion protein intermediate. Such TSEs typically include the human diseases Creutzfeld-Jacob disease (CJD), variant Creutzfeld-Jacob disease (vCJD), Kuru, fatal familial insomnia and Gerstmann-Straussler-Scheinker syndrome. Non-human TSEs include bovine spongiform encephalopathy (BSE), scrapie, feline spongiform encephalopathy, chronic wasting disease, and transmissible mink encephalopathy. Given that vCJD is currently understood to be a human form of BSE, it is apparent that certain TSE agents are capable of crossing the species barrier and that novel TSEs from non-bovine sources could become evident in future.

The proteolytic enzyme of the invention is typically a protease but can be suitably any biological polymeric molecule capable of catalysing cleavage of a polypeptide chain.

It is a feature of the invention that the proteolytic enzyme is a thermostable enzyme, that is, that it demonstrates optimal biological activity at temperatures in excess of the normal mammalian body temperature of 37°C. In embodiments of the invention the enzyme is thermally stable and biologically active, and inactivation is carried out, at temperatures equal to or greater than 40°C; preferably in the range of 50°C to 120°C; and more preferably where the temperature is between 55°C and 85°C. In a specific embodiment of the invention the temperature is about 60°C. In a further specific embodiment the temperature is about 80°C.

Thermostable proteolytic enzymes suitable for use in the methods and compositions of the invention are obtainable from a number of sources such as thermophilic bacteria and archaea. In one embodiment of the present invention the thermostable proteolytic enzyme is isolated from thermophilic bacteria, hyperthermophilic bacteria



and archaea. Suitable organisms for extraction of proteolytic enzymes for use in the invention include *Thermotoga maritima*; *Thermotoga neopolitana*; *Thermotoga thermarum*; *Fervidobacterium islandicum*; *Fervidobacterium nodosum*; *Fervidobacterium pennivorans*; *Thermosipho africanus*; *Aeropyrum pernix*; *Thermus flavus*; *pyrococcus spp.*; *Sulfolobus solfataricus*; *Desulfurococcus*; *Bacillus thermoproteolyticus*; *Bacillus stearo-thermophilus*; *Bacillus sp. 11231*; *Bacillus sp. 11276*; *Bacillus sp. 11652*; *Bacillus sp. 12031*; *Thermus aquaticus*; *Thermus caldophilus*; *Thermus sp. 16132*; *Thermus sp. 15673*; and *Thermus sp. Rt41A*.

- 10 The aforementioned organisms are not the only sources of thermostable proteases. Indeed, some organisms that are not considered to be truly thermophilic can also express thermostable proteolytic enzymes. Such organisms are commonly termed thermodurable in that although they do not choose to live in conditions of high temperature, they can withstand high temperatures for limited periods. A number of
- 15 *Bacillus* species fall in the category of thermodurability and are known to produce thermostable subtilisin-type proteases.

- The pH at which the inactivation is performed can range from acid to alkaline, but is typically in the region of pH 8-13, preferably pH greater than 9 and more preferably
- 20 around pH 12. Similarly, the thermostable protease is active in a pH range from acid to alkaline, but typically is optimally active in the region of pH 8-13, and preferably pH greater than 9 and more preferably around pH 12.

- In an example of the invention in use, the proteolytic enzyme is extracted from a
- 25 culture of the thermophilic bacteria or archaea. The culture is suitably maintained under the optimal conditions for the organism typically within a bioreactor. Thus, a continuous source of the organism can be maintained, allowing proteolytic enzyme to be obtained whenever needed.

- 30 Alternatively, in an example of the invention in use described in more detail below, the gene encoding a thermostable proteolytic enzyme is isolated from the source organism, *Bacillus thermoproteolyticus*. The gene is used to generate a recombinant expression construct, typically a plasmid, which is transformed into a host organism, *Escherichia coli*. The transformed *E. coli* is grown in a bioreactor and when at an
- 35 appropriate cell density the expression of the plasmid construct is initiated and the proteolytic enzyme harvested using standard methods. The recombinant expression route allows for the production of the proteolytic enzyme product under less extreme

conditions of temperature than would be required for the original source organism.

5 The recombinant route is further advantageous in that it allows for the genetic manipulation of recombinant thermostable protease genes in order to increase thermal stability or biological activity or for some other purpose. Thus, the activity of a thermostable proteolytic enzyme can be readily optimised for use in the methods and compositions of the invention.

10 Proteases are proteolytic enzymes that are carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "protease" means a naturally-occurring protease or a recombinant protease. Naturally-occurring proteases include  $\alpha$ -aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallo-carboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and  
15 acid proteases are included, as well as endo and exo-proteases.

The present invention includes the use of protease enzymes, for example naturally occurring carbonyl hydrolases or non-naturally occurring carbonyl hydrolase variants (protease variants). The protease enzymes useful in this invention exhibit a greater  
20 hydrolytic activity than proteinase K. In the context of this invention, the hydrolytic activity of the subtilisins was measured and comparable assays include, but are not limited to those described in Proteolytic enzymes, Practical Approach (Ed. by Beynon, RJ and Bond, JS, Oxford University Press, New York, Oxford, pp. 25-55 (1989); and the digestion of PrP-res proteins (Raymond, et al, Nature, 388:285-288  
25 (1997). For example, the enzymatic activity of subtilisins could also be measured by using chromogenic substrates. Incubation of proteases with these substrates could result in the cleavage of the substrate and liberation of p-nitroaniline that is detected spectrophotometrically at 405 nm. Other exemplary methods of analyzing the subtilisins are by using the substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (0.8  
30 mM in 20 mM sodium phosphate buffer, pH 8.5 or 0.8 mM in 20 mM Britton-Robinson buffer, pH 8.5). The incubation is carried out at 25° C. and followed spectrophotometrically for 4 min. The concentration of the protease is chosen so that the liberation of p-nitroaniline is linear during the whole analysis.

35 Proteases useful in the practice of this invention include all those disclosed in US Patent No. 6,312,936, the contents of which are hereby incorporated by reference, e.g. those proteases found in *Bacillus amyloliquefaciens* (SEQ. ID NO:7), *Bacillus*

*subtilis* (SEQ. ID. NO:8), *Bacillus licheniformis* (SEQ.ID.NO:9 ) and *Bacillus lentus* (SEQ.ID.NO:10 ). Another *Bacillus lentus* useful in the practice of this invention is the DSM 5483. The hydrolase variants may also have a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. Specifically, such protease variants have an amino acid sequence not found in nature, which is derived by substitution of a plurality of amino acid residues of a precursor protease with different amino acids. The precursor protease may be a naturally-occurring protease or a recombinant protease.

The protease variants useful herein encompass the substitution of any of the nineteen naturally occurring L-amino acids at the designated amino acid residue positions. Such substitutions can be made in any precursor subtilisin (procaryotic, eucaryotic, mammalian, etc.). Throughout this application reference is made to various amino acids by way of common one - and three-letter codes. Such codes are identified in Dale, M.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

The protease variants useful herein are preferably derived from a *Bacillus subtilis*. More preferably, the protease variants are derived from *Bacillus amyloliquefaciens* subtilisin, *Bacillus lentus* subtilisin and/or subtilisin 309.

Subtilisins are bacterial or fungal proteases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases, the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 15

herein. Generally and for purposes of the present invention, numbering of the amino acids in proteases corresponds to the numbers assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 13.

5 "Recombinant subtilisin" or "recombinant protease" refer to a subtilisin or protease in which the DNA sequence encoding the subtilisin or protease is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring amino acid sequence. Suitable methods to produce such modification, and which may be  
10 combined with those disclosed herein, include those disclosed in US Patent RE 34,606, US Patent 5,204,015 and US Patent 5,185,258, U.S. Patent 5,700,676, U.S. Patent 5,801,038, and U.S. Patent 5,763,257.

15 "Non-human subtilisins" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which subtilisin and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp.

20 A "protease variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor protease". The precursor proteases include naturally-occurring proteases and recombinant proteases. The amino acid sequence of the protease variant is "derived" from the precursor protease amino acid sequence by  
25 the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor protease rather than manipulation of the precursor protease enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as  
30 well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

One embodiment of the present invention utilizes protease variants having at least one modification of an amino acid position corresponding to positions 27, 76, 87,  
35 101, 103, 104, 123, 159, 222, 232, 236, 245, 248, 252, and 274 of *Bacillus amyloliquefaciens* subtilisin. Exemplary embodiments and/or combinations contemplated by the inventors include Y217L; K27R/V104Y/N123S/T274A;

N76D/S103A/V104I;S101G/S103A/V104I/G159D/A232V/Q236H/Q245R/N248D/  
N252K. Other embodiments include at least one modification of the precursor  
protease made to at least one position corresponding to positions 120, 167, 170,  
194, 195, and 235 of *Bacillus amyloliquefaciens*. Exemplary embodiments include  
5 combinations selected from G195E/M222A; M222S; Y167A/R170S/A194P;  
D36\_N76D/H120D/G195E/ K235N. Still another embodiment includes at least one  
modification at an amino acid position corresponding to positions selected from the  
group consisting of 3, 4, 99, 101, 103, 104, 159, 194, 199, 205, 217 of *Bacillus*  
*lentus*. Exemplary embodiments include combinations selected from  
10 S 9 9 D / S 1 0 1 R / S 1 0 3 A / V 1 0 4 I / G 1 5 9 S ;  
S99D/S101R/S103A/V104I/G159S/S3T/V4I/A194P/V199M/V205I/L217D and  
S99D/S101R/S103A/V104I/G159S/S3T/V4I/V205I/L217D of a mature *Bacillus lentus*  
DSM 5483 alkaline protease. These amino acid position numbers refer to those  
assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in  
15 Fig. 13. The invention, however, is not limited to the mutation of this particular  
subtilisin but extends to precursor proteases containing amino acid residues at  
positions which are "equivalent" to the particular identified residues in *Bacillus*  
*amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the  
precursor protease is selected from a *Bacillus amyloliquefaciens* or a *Bacillus lentus*  
20 subtilisin, the substitutions are made at the equivalent amino acid residue positions  
in *B. lentus* corresponding to those listed above.

A residue (amino acid) position of a precursor protease is equivalent to a residue of  
*Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in  
25 position in either primary or tertiary structure) or analogous to a specific residue or  
portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same  
or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a  
30 precursor protease is directly compared to the *Bacillus amyloliquefaciens* subtilisin  
primary sequence and particularly to a set of residues known to be invariant in  
subtilisins for which sequence is known. For example, Fig. 14 herein shows the  
conserved residues as between *B. amyloliquefaciens* subtilisin and *B. lentus*  
subtilisin. After aligning the conserved residues, allowing for necessary insertions  
35 and deletions in order to maintain alignment (i.e., avoiding the elimination of  
conserved residues through arbitrary deletion and insertion), the residues equivalent  
to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens*

subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained. Siezen et al. (1991) Protein Eng. **4(7)**:719-737 shows the alignment of a large number of serine proteases. Siezen et al. refer to the grouping as subtilases or subtilisin-like serine proteases.

For example, in Fig. 15, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* (carlsbergensis) and *Bacillus lentus* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 14.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other subtilisins such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred protease precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 15A and 15B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

"Equivalent residues" may also be defined by determining homology at the level of tertiary structure for a precursor protease whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor protease and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the protease in question to the *Bacillus amyloliquefaciens*

subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R_{factor} = \frac{\sum_h |F_o(h)| - |F_c(h)|}{\sum_h |F_o(h)|}$$

5

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor protease which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor protease (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to US Patent 5,182,204, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution are conserved residues whereas others are not. In the case of residues which are not conserved, the substitution of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such substitutions should not result in a naturally-occurring sequence. The protease variants of the present invention include the mature forms of protease variants, as well as the pro- and prepro-forms of such protease variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the protease variants.

A second aspect of the invention provides for a method of sterilising apparatus, comprising the step of exposing the apparatus to a solution comprising a thermostable proteolytic enzyme.

The term "sterilising" is commonly understood to mean the procedure by which living organisms are removed from or killed in a substrate, such as a piece of equipment or a solution. In the present case the TSE agent, or prion, is not technically considered to be a living organism, in the sense that a bacterium or virus is, because it does not apparently contain any genetic material. However, the transmission of the TSE pathogenic agent between animals does result in disease. Thus, the term "sterilising" as used herein is applied to the procedure by which both pathogenic agents (such as TSE agents) and living organisms are rendered non-infective or removed from or killed in a substrate.

In a preferred embodiment, the method of the invention comprises maintaining the sterilising solution at a temperature below 100°C, preferably at least 45°C and more preferably between 45°C and 85°C. The pH of the sterilising solution can range from acid to alkaline, but is typically in the region of pH 8-13, at least pH 9 and more preferably around pH 12. Similarly, the thermostable protease is active in a pH range from acid to alkaline, but typically is optimally active in the region of pH 8-13, at least pH9 and more preferably around pH 12.

In specific embodiments of the invention the sterilising solution is applied to the apparatus as a spray. The advantage of this mode of application is that, larger surface areas of apparatus, operating tables or even walls of rooms (for example in abattoirs) can be treated with the sterilising solution of the invention. Typically the solution will be heated to an optimal temperature, for example between 60°C to 80°C, before being sprayed onto the surface that is to be sterilised, that surface being optionally heated in advance.

Alternatively, the apparatus is immersed in the sterilising solution for a predetermined period of time. Again, the temperature of the solution is typically optimised prior to immersion of the contaminated apparatus. It is optional to include ultrasonication means in the immersion bath to enable ultrasonic cleaning to occur at the same time as treatment with the sterilising solution of the invention.

In a third aspect, the invention provides for a method of sterilising material, comprising exposing said material to a first solution comprising a thermostable proteolytic enzyme; and then exposing the apparatus to at least a second solution comprising a second thermostable proteolytic enzyme. In use, the material is typically apparatus, surgical or meat rendering equipment, or TSE infected biological



waste.

By dividing the sterilisation method into at least two, and optionally more, successive steps the conditions in each step can be optimised to ensure maximum inactivation of any TSE agent present. Thus, the temperatures and/or pH of successive steps can be different. In specific embodiments of the invention the proteolytic enzymes in the first and second (and optionally more) solutions are the same, or are different.

In a fourth aspect the invention provides for a composition for inactivating a TSE agent, comprising a thermostable proteolytic enzyme.

Typically, the composition of the invention further comprises a buffering agent. In a specific embodiment of the invention the buffering agent has a  $pK_a$  of between 8 and 13. Alkaline buffers suitable for use in the method of the invention include 4-cyclohexylamino-1-butanefulfonic acid (CABS) which has a  $pK_a$  of 10.7 at 25°C, and 3-cyclohexylamino-1-propanesulfonic acid (CAPS) which has a  $pK_a$  of 10.4 at 25°C.

Alternatively, the composition of the invention comprises sufficient sodium hydroxide or other alkaline agent to adjust the pH of the composition to alkaline, preferably to at least pH 9 and more preferably around pH 12. Addition of 1M sodium hydroxide to the composition of the invention, using a pH probe calibrated using Universal standards, is generally sufficient to set the pH of the composition to around 12.

Further provided by the invention is apparatus for inactivating a TSE agent comprising:

- a. a chamber for receiving contaminated material;
- b. means for controlling the temperature of the chamber; and
- c. a thermostable proteolytic enzyme active at alkaline pH, located within the chamber, the chamber optionally containing a solution of the thermostable proteolytic enzyme at a temperature of 45°C to 85°C.

Further aspects of the invention provide for uses of the aforementioned compositions for the inactivation of TSE agents.

An advantage of the invention is its use in degrading TSE and similar agents, and it has been found in operation of particular embodiments of the invention that TSE-contaminated material has been successfully decontaminated using a combination

of elevated temperature of around 50°C to 70°C and alkaline pH of around 9 to 12, with a thermostable, alkophilic proteolytic enzyme. Whilst it is on occasion possible to achieve some decontamination by extremely high temperature alone, it is of significant benefit to be able to inactivate TSE whilst avoiding extreme conditions, such as extremes of temperature, which lead to damage to the equipment being decontaminated

In a separate, though related, aspect of the present invention, the problem of detecting infective material is addressed. If it were possible to test an item of equipment for contamination either prior to or after carrying out a sterilisation process, then this would be of significant utility.

An anti-prion antibody, mAb 6H4 is available commercially from Prionics, Switzerland. This antibody can be used to detect prion protein, detected typically using a second antibody conjugated to a detectable marker, which second antibody binds to the first.

A difficulty that has been discovered by the present inventors is that binding of this antibody to equipment suspected of being contaminated with prion, or equipment that is suspected to be contaminated but which has been subjected to treatment intended to destroy the prion, does not correlate with infectivity. It has, for instance, been discovered by the inventors that prion-infected mouse brain homogenate, digested with protease, and run on SDS-PAGE, then probed with anti-prion antibody, shows a negative result, that is to say absence of antibody binding. This material nevertheless retains infectivity.

A further object of the invention is to provide methods and reagents for identifying infective prion material and for determining when infective material has been removed by treatment.

Accordingly, a further aspect of the invention provides a method of examining prion-infected or suspected prion-infected material and determining whether dimers of prion protein are present.

This aspect of the invention is based upon a correlation between presence of dimers of prion protein and presence of infectivity. As the prion protein can exist in a number of different glycosylation states, references to dimer is intended to include

dimers of the protein whether one or other or both is in any of a number of different possible glycosylation states. Reference to dimer is also intended to include reference to dimers of fragments of prion protein, those fragment-containing dimers retaining infectivity. Proteolytic treatment of the prion can result in removal of part of the protein sequence, leaving behind a residue which in dimer form retains infectivity, and these fragment dimers as well as prion-prion fragment dimers and fragment heterodimers are included within the definition of dimer in the present aspect of the invention.

10 The method enables detection of presence of dimer and hence enables detection of presence of infective material in cases where previous tests, designed to identify whether the monomer is present, would have given a negative result whereas in fact infectivity remained in that particular sample being tested. Hence, one advantage of the method is that at least some of the previous false negative results are  
15 eliminated. Avoiding these false negatives is clearly of significant value in testing alleged contaminated material as well as in testing material after treatment intended to remove infectivity.

As set out in more detail in an example below, prion dimers can be detected by using  
20 an antibody that binds to a prion monomer, using a second antibody, which has been labelled, to bind to the anti-prion antibody, and determining whether a protein is identified having approximately twice the molecular weight of the expected molecular weight of the prion monomer. As mentioned, the prion can be glycosylated in a number of different positions, typically 0, 1 or 2 positions, so when Western blotting  
25 is used, a number of bands may be seen on the resultant blot, corresponding to protein molecules having different glycosylation patterns. A known monomer is expected to have a molecular weight of about 33 - 35 kDa at its full length, however, it is routinely observed on SDS-PAGE gels and immunoblots as a limited proteolytic cleavage product known as "PrP 27-30". Correspondingly, the dimer is expected to  
30 be found at that part of the blot corresponding to about 54 - 70 kDa.

The dimer forms may be probed using an antibody specific to the dimer, that is to say an antibody which binds to the dimer but substantially does not bind to the monomer form of the prion. This antibody may itself be labelled or may be probed  
35 using a secondary labelled antibody.

A preferred method of this aspect of the invention thus comprises a method of

detecting prion infectivity comprising detecting prion dimer in a sample.

The invention additionally provides reagent for detecting prion dimers, and thus a further embodiment of the invention lies in an antibody specific for prion dimer, which antibody binds prion dimer but substantially does not bind prion monomer. This antibody optionally is labelled, and in an example described below the label is horseradish peroxidase; other suitable labels are alkaline phosphatase, beta galactosidase and D-biotin, though any suitable label may be used.

10 An alternative reagent for detection of presence of dimer comprises an antibody that binds to both monomer and a dimer and a molecular weight standard, such as one that corresponds to the molecular weight of the dimer. These are suitably used in combination with, for example, Western blotting techniques, to identify a protein having a molecular weight approximately twice that of the prion monomer. All these reagents are suitably incorporated into prion dimer detection kits.

To make an antibody selective for prion dimer, an animal is immunised with prion dimer and then serum extracted from the animal is run on a prion dimer column to identify antibodies that bind the dimer. These are then tested for cross-reactivity with prion monomer, with cross-reacting antibodies removed. The removal can be effected using a column loaded with prion monomer, the antibodies that emerge therefrom then being tested for absence of cross-reactivity.

Isolated prion dimer forms a further embodiment of the invention. This isolated material can be obtained simply by cutting out a portion of the SDS-PAGE gel used to resolve prion dimers. Alternatively, other separation techniques can be used to extract the prion dimer from homogenised prion-infected mouse brain. Antibodies that bind to the prion monomer and which are cross reactive can be used to confirm that the material thus obtained is prion dimer and not other protein of the same molecular weight.

As set out in more detail in the examples, prion strain 301V, a mouse passaged isolate, derived from a Holstein-Fresian cow terminally ill with BSE is used as an example of a prion strain. Infection is known to be produced by intracerebral inoculation and the incubation period required for the onset of clinical symptoms is remarkably uniform. That is to say, providing that the dose of infectious agent is sufficient then the classic signs of disease will appear at a defined time post-

inoculation (in VM mice this is 120 days). For obvious reasons no-one has tested these properties on humans, however, the mouse bioassay is regarded as the closest available model and therefore a good indicator of BSE infection in man.

- 5 Using the Western blot detection system, in an example below, the monomer is apparently completely removed by protease digestion. Under these conditions, however, samples which were subsequently used *in vivo* did not prevent infection and may even have enhanced its onset. There must, therefore, be another source of infection other than the monomer. Since the monomer can be removed (or at  
10 worst dramatically reduced in concentration) this indicates a primary role in infectivity for the dimer - either alone or in combination with the monomer.

The second aspect of the invention thus also provides dimer removal using protease degradation according to the first aspect which is optionally enhanced by  
15 environmental conditions - high temperature, extremes of pH, and/or the use of detergents (e.g. SDS). As well as using single enzyme treatments, combinations of proteases and/or other enzymes can be used. For example, better degradation of the infective agent may be achieved by the addition of lipases, peptidases, glycosylases, nucleases and other enzymes.

20 To confirm dimer removal, a dimer cross-reactive antibody can be used in conjunction with a suitable detection system, one example being a sensitive *in vitro* detection system currently available from Invitrogen, referred to as Western Breeze, to confirm removal prior to further mouse bioassays.

25 The methods and compositions of specific embodiments of the inventions are described in more detail below and are illustrated by the accompanying drawings in which;

30 Fig. 1 shows the effect of temperature on a protease M digest of mouse brain homogenate (mbh);

Fig. 2 shows the effect of pH on a protease M digest of mbh;

35 Fig. 3 shows a *Bacillus thermoproteolyticus* Rokko digest of mbh;

Fig. 4 shows the effect of sodium dodecyl sulfate (SDS) on Rokko digest of

mbh;

Fig. 5 shows and SDS-PAGE of mbh;

5 Fig. 6 shows and immunoblot of mbh;

Fig. 7 shows a protease G, R and C digest of mbh;

Fig. 8 shows an immunoblot of the digest in Fig. 7;

10

Figs 9 to 12 show blots of BSE (301V)-infected mouse brain homogenate, to illustrate correlation of infectivity with prion dimer, and as further explained in the examples below;

15 Fig.13 A-C depict the DNA (SEQ.ID.NO:1) and amino acid sequence (SEQ.ID.NO:2) for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene.

20 Fig.14 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN)' and *Bacillus lentus* (wild-type).

25 Figs. 15A and 15B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN') (SEQ.ID.NO: 7). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (SEQ.ID.NO: 8). The third line depicts the amino acid sequence of subtilisin from *B. licheniformis* (SEQ.ID.NO: 9). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (SEQ.ID.NO:10). The symbol \* denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

30

Fig. 16 shows an MC-A, MC-3 and MC-4 digest of mbh.

Fig. 17 shows a comparison of MC-A, MC-3 and MC-4 mbh digests with a Properase mbh digest.

35

Fig. 18 also shows a comparison of MCA, MC-3 and MC-4 mbh digests with a Properase mbh digest.

Fig. 19 shows a temperature profile of MC-3.

Fig. 20 shows detection of MC-A, MC-3 and MC-4 mbh digests with PAb2.

5 Fig. 21 shows MC-3 dilutions at pH 10 and pH 12.

Fig. 22 shows a comparison of MC-A, MC-3 and MC-4 mbh digests with a Proteinase K mbh digest.

## 10 **EXAMPLES**

### **Example 1**

#### **VM Mouse Colony and Incubation with BSE (301V) agent**

15

Studies on the inactivation of the TSE agent, BSE strain (301V), required the establishment of a mouse breeding colony for the generation of both uninfected and infectious brain homogenate (mbh) and its subsequent titration and bioassay. The VM mouse strain selected for use in the study was obtained from Dr David  
20 Taylor (Institute of Animal Health, Edinburgh). Six pairs were introduced into a dedicated room within an animal facility. Mice were screened for their health status and a breeding programme initiated.

25

BSE (301V) infectious mouse brain (IAH, Edinburgh) was prepared for inoculation by crude homogenisation followed by passage of the brains through increasingly fine gauge luer-locked needles (from 21G-27G) to and from a contained safety syringe into a closed septum-topped vial. This procedure was carried out in a validated safety cabinet within a containment level 3 (CL3) laboratory immediately prior to intracerebral inoculation of the VM mice. The anaesthetised  
30 (alphadolone/alphaxalone) mice were inoculated intracerebrally via 26 gauge needles with 20ml of the mouse brain homogenate preparation. Forty-nine out of fifty mice survived this procedure. These were retained to allow incubation of the agent and the generation of the required quantity of high-titre infectious material.

35

#### **Biomass Production**

A wide variety of organisms were chosen for the production of biomass in order to

provide as broad a selection of thermostable proteolytic enzymes as possible. Organisms selected ranged from those growing optimally at moderately thermophilic temperatures (50°C) through to extremely thermophilic temperatures (100°C) and included members of both the Archaea and the Bacteria. Thermophiles were also  
5 selected to cover a wide range of growth pH, encompassing pH optima from pH2.5 to pH11.5. The majority of organisms were grown in batch culture, however, where the growth requirements of the organism were particularly fastidious, continuous culture was used (Raven and Sharp (1997) *Applied Microbial Physiology: A Practical Approach*, Ch. 2, Eds. Stanbury and Rhodes, OUP pp.23-52). Depending on the  
10 biomass yield of the organism being grown, batch culture volumes of between 20L and 120L were employed to achieve the desired amount of cell paste. The continuous culture system utilised either a 2L or 5L working volume gas lift bioreactor constructed entirely of glass and PTFE. More prolific organisms such as *Bacillus* spp., and *Thermus* spp., were pre-screened to select those with high levels of  
15 protease activity prior to their culture on a larger scale. A quick and sensitive fluorometric protease assay utilising microtitre plates was adopted for this purpose to permit high through-put screening (EnzChek™, Molecular Probes, Leiden, Netherlands).

20 Culture biomass was harvested by continuous centrifugation (Contifuge Stratos™, Kendro Laboratory Products, Bishop Stortford, UK) and stored at -80°C. Culture supernatants were concentrated with a 10KDa cut off tangential flow filter (Pall filtration, Portsmouth, UK). Proteins were precipitated with ammonium sulphate (90% saturation) and stored at -80°C.

## 25 Protein Purification

A rapid protease screening and purification technique was required in order to process all of the crude protein preparations after the biomass production stage. A  
30 dye-ligand affinity chromatography system was used for this purpose (PIKSI M™, Affinity Chromatography Ltd., Isle of Man, UK). Initially, each crude ammonium sulphate precipitate was dissolved in buffer and passed through a desalting column. Each sample was then loaded onto the PIKSI M test kit, which contained 10 different affinity ligands. Fractions were then assayed for protease activity to determine the  
35 most suitable matrix for purification of the protease, either by positive binding of the target molecule and then elution, or by negative binding of contaminants. The purification was then scaled up using the same affinity matrix in conjunction with an



FPLC system (Amersham-Pharmacia Biotech, Amersham, UK). By combining this technique with the fluorometric protease assay, the rapid screening of many fractions could be undertaken.

- 5 The fluorometric protease assay utilises casein derivatives that are heavily labelled with green fluorescent BODIPY FL in which the conjugates' fluorescence is almost totally quenched. Protease catalysed hydrolysis releases the highly fluorescent label and the resultant fluorescence can be measured on a fluorometric microplate reader (Labsystems Fluoroscan II™). The increase in fluorescence is proportional to  
10 protease activity and was compared with that of a standard protease (Protease X, Sigma-Aldrich, Poole, UK).

### Protease Characterisation

- 15 A range of thermostable proteases were analysed (see Table 1). Direct characterisation of activity was carried out using the closest non-infectious analogue to BSE (301V) - infectious mouse brain homogenate available as substrate, i.e. normal VM mouse brain homogenate. Initial digests of total uninfected mouse brain homogenate (mbh) were performed over thirty minutes at 60°C and at pH7.0. The  
20 samples were then boiled under reducing conditions and analysed by SDS-PAGE on pre-cast NuPage 4 - 12% Bis-Tris gels (Novex™, San Diego, US). Gels were fixed using standard procedures and the proteins visualised using the Novex colloidal blue staining kit. The results showed both the levels of activity of the proteases against the mbh substrate under these conditions and also the level of purity of  
25 individual proteases. Protease concentrations used in the assays were based on total protein concentration, therefore, a wide range of activities was observed from limited to complete digestion.

- Activity profiles showing the full effect of pH and temperature on mbh digestion were  
30 then produced for each enzyme. Several enzymes showed extensive activity over the complete range of conditions (pH 2 - 12 and 50 - 100°C). In general, however, complete digestion of mbh was only achieved over a narrower range indicative of the enzymes' pH and temperature optima. Figs. 1 and 2 show the activity profiles for a sample protease, *Bacillus* protease M. As can be seen the enzyme is moderately  
35 thermophilic and gives complete digestion at temperatures up to 70°C. Similarly the pH profile indicates a preference for neutral or alkaline conditions.

## Protease Testing

Several enzymes did not give complete digestion of mbh even when incubation times were increased to 24 hours. This may have simply been due to low protease activity, however, several highly purified and concentrated enzymes still only produced partial digests. In these reactions little enzyme-substrate interaction appeared to be occurring. It was considered that this could be due to non-proteinaceous material, e.g. lipids, surrounding the substrate and preventing interaction, however, pre-treatment with lipases and other enzymes had no observable effect (data not shown). A second possibility considered was the effect of repulsive surface charges between proteases and the substrate. One enzyme in particular, purified from *Bacillus thermoproteolyticus* Rokko, consistently produced poor digestion of mbh even at high concentrations. This enzyme was known to remain active in the presence of high levels of detergents, therefore, digests were performed with the addition of SDS in an attempt to overcome this effect. Figures 3 and 4 show *B.thermoproteolyticus* Rokko digests of mbh in the absence and presence of SDS. Figure 3 clearly indicates that under standard conditions there is little difference in digestion even as the protease concentration is increased to 20mg.ml<sup>-1</sup>. On addition of SDS (lanes 2-5), no protein bands, except the protease itself, are visible indicating a complete digest. Tested proteases could, therefore, be divided simply into three categories: (i) those able to give total mbh digestion in the absence of detergent, (ii) those able to give total mbh digestion in the presence of SDS and (iii) those unable to digest the substrate fully. Proteases in categories (i) and (ii) were selected for immediate further study, while those in category (iii) were either rejected or assumed to be required in greater quantity and/or higher purity.

## Western Blotting of Mouse Brain Homogenate

Mbh proteins were transferred onto nitrocellulose and blocked overnight in PBS-Tween (PBST) + 3% skimmed milk powder. The membrane was washed (x3 in PBST) and incubated for 1 hour with 6H4 anti-human recombinant PrP monoclonal antibody (Prionics, Zurich, Switzerland). After a second washing step, anti-mouse HRP-conjugate was added and the membrane incubated for 1 hour. Washing was repeated and the antibody reaction visualised by addition of TMB (Harlow and Lane (1988), Antibodies: A Laboratory Manual, Cold Spring Harbor Press).

Figures 5 and 6 show an SDS-PAGE and an immunoblot of undigested mbh

respectively. Although there is some non-specific background, dark bands indicating the presence of mouse prion can clearly be seen at the expected molecular weight (~33-35kDa). Distinct bands are also visible at ~66-70kDa, which may correspond to prion dimers previously reported (Safar *et al.* (1990) Proc. Natl. Acad. Sci. USA, 87:pp6373-6377).

Western transfer and immunoblotting were used to confirm that no immunoreactive fragment of mouse PrP<sup>C</sup> remained after digestion. Figure 7 shows mbh digested to completion with three closely related thermostable proteolytic enzymes (Proteases G, R and C), as assessed by SDS-PAGE. In the corresponding immunoblot (Figure 8) only 2 bands are visible, both with an apparent MW of ~23kDa. The strong band in lane 9 corresponds to recombinant mouse PrP (+ve control), whilst the weak band in lane 4 appears to be due to a slight reaction with the heavily loaded enzyme preparation. In this case, complete loss of PrP<sup>C</sup> immunoreactivity is still apparent since no band is observable in lane 3.

### **Preparation and Titration of Infectious Mouse Brain Homogenate**

Eighty days post challenge onward, mice were subject to daily clinical scoring to detect clinically affected mice as early as possible. A single mouse died of an unknown cause prior to this period. The remainder exhibited the expected disease progression and were sacrificed between 110 and 130 days post challenge. The brains were removed aseptically and stored frozen until required. Forty-eight BSE (301V) infectious VM mouse brains were homogenised in four volumes of PBS within a contained homogeniser then passed sequentially through increasingly fine gauge needles (21G to 26G) until free flowing. A sample with a further 2-fold dilution (1:9 mouse brain: PBS) was prepared for titration of infectivity. Over 800 x 0.1ml aliquots of BSE (301V) infectious mouse brain homogenate were prepared. These procedures were again carried out under rigorous class III containment, including the wearing of positive pressure respirators.

Groups of 25 eight week old VM mice received titration doses of the infectious mouse brain homogenate preparation at 10 fold dilutions from  $10^{-1}$  –  $10^{-8}$ . A further group of 25 mice were challenged with uninfected mouse brain homogenate as a control. All mice were inoculated under anaesthetic using 26G x 3/8" (0.95cm) needles with plastic sleeve-guards cut off 2mm below bevel in a Class 2 cabinet with the use of an injection guard. The mice were then left to incubate the BSE (301V)

agent for extended periods some in excess of a year. The initial titre of the infectious mouse brain homogenate preparation was established retrospectively once all incubations (clinical monitoring at 80 days onwards) were complete.

## 5 Dimer detection in digested mouse brain

BSE (301V)-infected mouse brain homogenate was digested at neutral pH and 60°C for 30 minutes with protease. Total protein digests were run on SDS-PAGE and transferred by Western blotting to nitro-cellulose membranes. These were cut into strips and probed with CAMR anti-prion antibodies (produced in rabbits). A second generic antibody (goat anti-rabbit) was conjugated to horseradish peroxidase and used with detection by TMB colorimetric substrate.

At the time, the expected result was that the results were the same as the control blot (number 7). - using the anti-prion antibody mAb 6H4 (from Prionics, Switzerland). In this control blot, there is seen the typical three-banded pattern (glycosylation states) for protease-digested infectious-conformation prion protein (PrP<sup>Sc</sup>).

However, the blots in this example did not show this pattern. Blot 1 uses a polyclonal antibody raised against a PPD-conjugated peptide corresponding to an N-terminal region of the prion molecule. Nothing is seen in the lanes. This section of the protein is susceptible to proteolysis, so it is not surprising to see nothing in the lanes (2 & 3) – see figure 9, blot 1 on left hand side. Lane 1 is a molecular weight marker.

Blot 2 has a second antibody raised against a peptide sequence further into the prion molecule. This shows at least 9 bands of varying intensity, approximately equidistant, at a molecular weight corresponding to a prion dimer with a range of glycosylation states – see blot 2 on figure 9.

Blot 3 antibody shows similar profile; blot 4 is also shown but its results are too poor quality to draw any conclusions – see blots 3 and 4 on figure 9.

Blots 5 and 6, shown on figure 10 with the control blot 7, again show the multi-banded pattern

## Dimer detection in digested mouse brain

The above example was repeated, and the results shown in figures 11 and 12.

5 Blot 1 shows molecular weight markers in lanes 1 and 5. Lane 2 is recombinant murine PrP showing recombinant murine PrP oligomers. Lane 3 shows lack of antibody response to protease-digested infectious mouse brain homogenate. Lane 4 is the antibody response in the undigested control.

10 Blot 2 is as above but shows the previous banding pattern in the protease digested sample.

Blot 3 shows the antibody 3 response. Here there is some response to recombinant murine PrP (lane 2). Lane 3 shows not only the multiple (dimeric PrP) banding pattern, but also some monomeric PrP response.

15 Blot 7 is the 6H4 mAb antibody control. Here there is good detection of recombinant murine PrP oligomers (lane 2). Lane 3 shows the heavily diglycosylated form of limitedly protease-treated PrP<sup>Sc</sup>, plus the more minor monoglycosylated and non-glycosylated forms typical of BSE (301V) strain. No 'dimer' detection is apparent.

## 20 **Preparation of antibodies including dimer preferential antibody**

In the examples, we have used 6 polyclonal antibodies. Of these, three detect the dimer alone and do not bind the monomer whereas one cross-reacts with both the monomer and the dimer.

25

The polyclonal sera were produced by immunisation of rabbits with synthesised prion mimetic peptides. These peptides were designed based on regions of high homology between human, mouse and bovine prion protein amino acid sequences.

30 The sequences producing the dimer-reactive antibodies were as follows:

CGGWGQPHGGC (Peptide2)

CGGYMLGSAMSRPIIHFGNDYEC (Peptide 3)

CVNITIKQHTVTTTTKGENFTETDC (Peptide 5)

35 CITQYQRESQAYYQRGASC (Peptide 6)

The peptides were synthesised with a cysteine at both ends (see above) and with a

cysteine at one end only. This method was used in order to present both the linear form and a loop structure of the antigen on the surface of the carrier protein.

5 The peptides were synthesised commercially and coupled to the carrier protein PPD (purified protein derivative), derived from an attenuated strain of the bacterium *Mycobacterium bovis*, which is lyophilised and used to conjugate to the peptide via a linker.

Anti-prion polyclonal antibodies were produced as follows:

10

A sample of pre-immune sera (~1ml) was collected from each of a group of Dutch rabbits.

15 The rabbits were injected with reconstituted freeze-dried Bacillus Calmette-Guerin (BCG) vaccine for intradermal use. A dose of 0.1ml of reconstituted BCG vaccine was given in two sites in the scruff of the neck of the rabbit.

After 4 weeks, 0.6mg of each peptide-PPD conjugate was measured (0.3mg of each of the 1 cysteine and 2 cysteine versions) and dissolved in 1ml of sterile 0.9% saline.

20 An equal volume of incomplete Freund's adjuvant was added and 0.75ml aliquots, of the resulting emulsion, were injected intra-muscularly into each hind limb and 0.25ml aliquots into two sites in the scruff of the neck per rabbit.

After 4 weeks a boost injection was given comprising of the peptide-PPD conjugates prepared as in step 3 and 4. The boost injections consist of four 0.25ml injections into the scruff of the neck of each rabbit.

25 7-14 days after the first boost injections, 4ml test bleeds were taken, the sera was assessed by ELISA for antibody titre.

A second boost injection was given 4-6 weeks after the first.

A third boost injection given 4-6 weeks later.

30 A 4ml test bleed was taken 6-8 weeks after the third boost injection and antibody titres determined by ELISA

A fourth boost injection given.

A 4ml test bleed was taken 7-14 days after the fourth boost injection and antibody titre determined by ELISA.

35 Terminal exsanguination was carried out and blood collected. The serum was separated by centrifugation and stored at -20°C.

Analysis of antibody titre was achieved using ELISA. The immunoassay plate was

coated with the same peptides conjugated to a different carrier protein (KLH) in order to differentiate the response to the peptide from the response to the carrier protein.

Three of the antibodies produced by immunisation of the synthetic peptide sequences described bind preferentially to the dimer form of the molecule.

Analagous steps may also be used to prepare a monoclonal antibody. This could be achieved using a method such as described in Antibodies – A Laboratory Manual, Ed Harlow and David Lane, 1988 (Cold Spring Harbor Laboratory).

## **Example 2**

### **Evaluation of proteases MC-A, MC-3 and MC-4**

Three new proteases, MC-A, MC-3 and MC-4, were assessed using infectious BSE (301V) mouse brain homogenate (mbh) dialysed to pH 2,4,6,8,10 and 12 respectively and digested at 50°C for 30 minutes. Total protein digests were run on SDS-PAGE and transferred by Western blotting to nitro-cellulose membranes.

The Western blots were detected with 6H4 and TMB colorimetric substrate, and the results shown in Figure 16.

Figure 16 demonstrates characteristic PrP<sup>Sc</sup> monomer bands present after digestion with the new proteases at pH 2-10. The monomer bands present after digestion with MC-3 at pH 2-10 appear very faint. Indeed, of the three proteases tested, MC-3 shows the greater reduction of monomer.

In addition, no PrP<sup>Sc</sup> monomer bands are present at pH 12 with any of the three proteases.

### **Comparison of new proteases with Properase**

The digestion of PrP<sup>Sc</sup> monomers using the three new proteases was compared to digestion using Properase, and the results shown in Figures 17 and 18.

Figure 17 demonstrates monomer digestion at pH 2-12 by MC-A, MC-3 and MC-4

at a temperature of 50°C, compared to Properase at 60°C. At 50°C, the three new proteases give improved monomer digestion compared to Properase at 60°C.

Figure 18 shows monomer digestion at pH 2-12 by MC-A, MC-3 and MC-4 at a temperature of 60°C, compared to Properase at 60°C. At 60°C, digestion of the monomers with the three new proteases is comparable to that using Properase.

### **Temperature Profiling of MC-3**

The effect of temperature on monomer digestion by MC-3 was investigated, with results shown in Figure 19.

Figure 19 shows increased temperature to result in increasingly incomplete digestion of mbh at low pH. However, at high pH (pH 10 and above), increased temperature leads to less distinct monomer bands. This suggests that at increased temperatures, high pH enhances digestion of monomer by MC-3. It is also noted that MC-3 at 50°C has a greater effect across the pH range than either MC-A or MC-4.

### **Detection with PAb2**

Mouse brain homogenates at pH 2-12 were digested at 50°C for 30 minutes and the corresponding Western blots detected with a chemiluminescent detection substrate, PAb2. The results are shown in Figure 20.

Figure 20 shows high molecular weight (HMW) bands present after digestion with each protease at pH 2-10. The HMW bands at pH 12 are much reduced with all three proteases. This implies that the HMW bands are proteinaceous, as they can be removed by proteases. These bands are thought to represent PrP<sup>Sc</sup> dimers.

### **MC-3 Dilutions at pH 10 and pH 12**

Figure 21 shows the results of MC-3 dilutions at pH 10 and pH 12.

At pH 10, monomer bands are seen at 1:20 dilution and HMW bands are seen across the dilution range. However, at pH 12, no monomer bands are apparent



and HMW bands are much reduced across the dilution range. This suggests high pH enables MC-3 to digest HMW dimer.

### **Comparison with Proteinase K**

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The ability of the three new proteases to digest monomer and HMW dimer was compared to Proteinase K, and the results shown in Figure 22.

10

The results indicate that all three new proteases are better at removing both the monomer and the HMW dimer than Proteinase K.

### **Summary of Results**

15

Of the proteases tested, MC-3 shows the greater reduction of the monomers. MC-3 is better at removing the monomer bands across the pH range than either Properase or Proteinase K. Unlike Properase or Proteinase K, the new proteases reduce the HMW bands at pH 12.

20

The invention thus provides detection and degradation of TSE infectivity.

**Table 1**

	Organism	Domain	Growth	T opt	pH opt
5	<i>Aeropyrum pernix</i>	Archaeon	Aerobe	95°C	7.0
	<i>Alicyclobacillus acidocaldarius</i>	Bacterium	Aerobe	65°C	3.5
	<i>Archaeoglobus fulgidus</i>	Archaeon	Anaerobe	85°C	6.5
	<i>Bacillus caldotenax</i> BT1	Bacterium	Aerobe	65°C	7.0
	<i>Bacillus pallidus</i>	Bacterium	Aerobe	65°C	9.0
10	<i>Bacillus stearothermophilus</i> L32-65	Bacterium	Aerobe	65°C	7.0
	<i>Bacillus stearothermophilus</i> LUDA T57	Bacterium	Aerobe	65°C	7.0
	<i>Bacillus thermoproteolyticus</i> Rokko	Bacterium	Aerobe	65°C	7.0
	<i>Bacillus</i> sp. 11231	Bacterium	Aerobe	65°C	7.0
	<i>Bacillus</i> sp. 11276	Bacterium	Aerobe	65°C	7.0
15	<i>Bacillus</i> sp. 11652	Bacterium	Aerobe	65°C	7.0
	<i>Bacillus</i> sp. 12031	Bacterium	Aerobe	65°C	7.0
	<i>Desulfurococcus</i> sp.	Archaeon	Anaerobe	85°C	6.5
	<i>Fervidobacterium pennivorans</i>	Bacterium	Anaerobe	70°C	8.5
	<i>Hyperthermus butylicus</i>	Archaeon	Anaerobe	95°C	6.5
20	<i>Pyrococcus furiosus</i>	Archaeon	Anaerobe	95°C	7.5
	<i>Pyrococcus horikoshii</i>	Archaeon	Anaerobe	95°C	7.0
	<i>Sulfolobus acidocaldarius</i> 98-3	Archaeon	Aerobe	75°C	2.5
	<i>Sulfolobus hakonensis</i>	Archaeon	Aerobe	75°C	2.5
	<i>Sulfolobus solfataricus</i> P1	Archaeon	Aerobe	75°C	2.5
25	<i>Sulfolobus solfataricus</i> P2	Archaeon	Aerobe	75°C	2.5
	<i>Thermobrachium celere</i>	Bacterium	Anaerobe	65°C	8.5
	<i>Thermococcus fumicolans</i>	Archaeon	Anaerobe	85°C	6.5
	<i>Thermus caldophilus</i> GK24	Bacterium	Aerobe	70°C	8.0
	<i>Thermus aquaticus</i> YT1	Bacterium	Aerobe	70°C	8.0
30	<i>Thermus</i> sp. 16132	Bacterium	Aerobe	70°C	8.0
	<i>Thermus</i> sp. 15673	Bacterium	Aerobe	70°C	8.0
	<i>Thermus</i> sp. Rt41A	Bacterium	Aerobe	70°C	8.0

**CLAIMS**

- 5 1. A method for inactivating a transmissible spongiform encephalopathy (TSE) agent comprising exposing the TSE agent to a thermostable proteolytic enzyme.
- 10 2. A method according to claim 1, comprising exposing the TSE agent to the thermostable protease at a temperature that is equal to or greater than 40°C.
- 15 3. A method according to claim 2, wherein the temperature is between 50°C and 120°C.
- 20 4. A method according to claim 3, wherein the temperature is between 55°C and 85°C.
- 25 5. A method according to claim 4, wherein the temperature is about 60°C.
- 30 6. A method according to claim 4, wherein the temperature is about 80°C.
7. A method according to any previous claim wherein the pH is neutral (pH 7.0).
8. A method according to claims 1-6, wherein the pH is acidic.
- 35 9. A method according to claims 1-6, wherein the pH is alkaline.
10. A method according to claim 9, wherein the pH is between 8 and 13.
11. A method according to claim 9, wherein the pH is about 12.
12. A method according to any previous claim wherein the TSE agent is a prion.
13. A method according to any previous claim, wherein the TSE agent is selected from the group consisting of Creutzfeld-Jacob disease; variant Creutzfeld-Jacob disease; Kuru; fatal familial insomnia; Gerstmann-Straussler-Scheinker syndrome; bovine spongiform encephalopathy; scrapie; feline spongiform encephalopathy; chronic wasting disease; and transmissible mink encephalopathy.

14. A method according to any previous claim wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
- 5 15. A method according to claim 14 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
- 10 16. A method according to claim 15 wherein the thermophilic organism is selected from the group consisting of *Thermotoga maritima*; *Thermotoga neopolitana*; *Thermotoga thermarum*; *Fervidobacterium islandicum*; *Fervidobacterium nodosum*; *Fervidobacterium pennivorans*; *Thermosipho africanus*; *Aeropyrum pernix*; *Thermus flavus*; *pyrococcus* spp.; *Sulfolobus solfataricus*; *Desulfurococcus*; *Bacillus thermoproteolyticus*; *Bacillus stearo-thermophilus*; *Bacillus* sp. 11231; *Bacillus* sp. 11276; *Bacillus* sp. 11652; *Bacillus* sp. 12031; 15 *Thermus aquaticus*; *Thermus caldophilus*; *Thermus* sp. 16132; *Thermus* sp. 15673; and *Thermus* sp. Rt41A.
- 20 17. A method of sterilising apparatus comprising exposing said apparatus to a solution comprising a thermostable proteolytic enzyme.
18. A method according to claim 17, wherein the solution is maintained at a temperature below 100°C.
- 25 19. A method according to claim 17, wherein the solution is maintained at a temperature of between 45°C and 85°C.
20. A method according to any of claims 17-19, wherein the solution has a neutral pH.
- 30 21. A method according to any of claims 17-19, wherein the solution has an acidic pH.
22. A method according to any of claims 17-19, wherein the solution has an alkaline pH.
- 35 23. A method according to claim 22, wherein the solution has a pH of between 8 and 13.

24. A method according to any of claims 17-23, wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
- 5 25. A method according to claim 24 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
26. A method according to any of claims 17-25, wherein the solution is applied to the apparatus as a spray.
- 10 27. A method according to any of claims 17-25, wherein the apparatus is immersed in the solution.
- 15 28. A method of sterilising apparatus, comprising exposing said apparatus to a first solution comprising a thermostable proteolytic enzyme; and then exposing the apparatus to at least a second solution comprising a second thermostable proteolytic enzyme.
- 20 29. A method according to claim 28, wherein the first and second proteolytic enzymes are the same.
- 30 30. A method according to claim 28, wherein the first proteolytic enzyme is different to the second proteolytic enzyme.
- 25 31. A method according to any of claims 28-30, wherein the pH of the first solution is different to the pH of the second solution.
32. A method according to any of claims 28-31, wherein the temperature of the first solution is different to the temperature of the second solution.
- 30 33. A composition for inactivating a TSE agent, comprising a thermostable proteolytic enzyme.
34. A composition according to claim 33, further comprising a buffering agent.
- 35 35. A composition according to claim 34, wherein the buffering agent has a  $pK_a$  of between 8 and 13.

36. A composition according to claim 33, further comprising sufficient sodium hydroxide to set the pH of the composition to around 12.
- 5 37. A composition according to claims 33-36, further comprising a detergent compound.
38. A composition according to claim 37, wherein the detergent is sodium dodecyl sulphate (SDS).
- 10 39. A composition according to any of claims 33-38, wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
40. A composition according to claim 39 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
- 15 41. Use of a composition according to claims 33-40 for sterilising material contaminated with a TSE agent.
- 20 42. Apparatus for inactivating a TSE agent comprising:
- a. a chamber for receiving contaminated material;
- b. means for controlling the temperature of the chamber; and
- 25 c. a thermostable proteolytic enzyme active at alkaline pH, located within the chamber.
43. Apparatus according to claim 42 comprising a solution of the thermostable proteolytic enzyme at a temperature of 45°C to 85°C.
- 30 44. A method of examining a sample infected with or suspected to be infected by prion protein, comprising detecting dimers of prion protein in the sample.
- 35 45. A method according to Claim 44 comprising determining whether the sample contains prion monomer, prion dimer, or mixtures of prion monomer and dimer.

46. A method according to Claim 44 or 45, comprising determining whether the sample contains a dimer of a fragment of a prion.
- 5 47. A method according to any of Claims 44 to 46, comprising probing the sample with an antibody specific to prion monomer, and determining whether the sample contains protein with a molecular weight corresponding to approximately twice that of prion monomer.
- 10 48. A method according to any of Claims 44 to 46, comprising probing the sample with an antibody specific to prion dimer.
49. A method of detecting prion infectivity, comprising detecting prion dimer in a sample.
- 15 50. An antibody, which is specific for prion dimer but does not bind to prion monomer.
51. A labelled antibody according to Claim 50.
- 20 52. A method of producing an antibody, comprising immunising an animal with a prion dimer, obtaining an extract therefrom which contains antibodies, and isolating from said extract antibodies which bind prion dimer.
- 25 53. A method according to Claim 52, comprising obtaining an antibody preparation containing antibodies which bind prion dimer, and removing from said preparation antibodies which also bind prion monomer.
54. Purified prion dimer.
- 30 55. A method according to any of Claims 1 to 32 wherein the thermostable proteolytic enzyme is a serine protease.
56. A method according to any of Claims 1 to 32 wherein the thermostable proteolytic enzyme is a subtilisin.
- 35 57. A method according to any of Claims 1 to 32 wherein the thermostable proteolytic enzyme is a subtilisin derived from *Bacillus* bacteria.

58. A method according to any of Claims 1 to 32 wherein the thermostable proteolytic enzyme is a subtilisin derived from *Bacillus amyloliquefaciens*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus subtilis* or is subtilisin PB92 or subtilisin 309..
- 5 59. A method according to any of Claims 1 to 32 wherein the thermostable proteolytic enzyme is a variant or fragment of any subtilisin of Claim 58.
- 10 60. A method according to any of Claims 1 to 32 wherein the thermostable proteolytic enzyme is MC-A, MC-3 or MC-4.
- 15 61. A composition according to any of Claims 33 to 40, or an apparatus according to Claims 42 or 43, wherein the thermostable proteolytic enzyme is a serine protease.
62. A composition according to any of Claims 33 to 40, or an apparatus according to Claims 42 or 43, wherein the thermostable proteolytic enzyme is a subtilisin.
- 20 63. A composition according to any of Claims 33 to 40, or an apparatus according to Claims 42 or 43, wherein the thermostable proteolytic enzyme is a subtilisin derived from *Bacillus bacteria*.
- 25 64. A composition according to any of Claims 33 to 40, or an apparatus according to Claims 42 or 43, wherein the thermostable proteolytic enzyme is a subtilisin derived from *Bacillus amyloliquefaciens*, *Bacillus lentus*, *Bacillus licheniformis*, or is subtilisin PB92 or subtilisin 309..
- 30 65. A composition according to any of Claims 33 to 40, or an apparatus according to Claims 42 or 43, wherein the thermostable proteolytic enzyme is a variant or fragment of any subtilisin of Claim 64.
- 35 66. A composition according to any of Claims 33 to 40, or an apparatus according to Claims 42 or 43, wherein the thermostable proteolytic enzyme is MC-A, MC-3 or MC-4.



## DEGRADATION AND DETECTION OF TSE INFECTIVITY

5

### ABSTRACT

10 A transmissible spongiform encephalopathy (TSE) agent is inactivated by exposing the TSE agent to a thermostable proteolytic enzyme at elevated temperature and at acid or alkaline pH. Following this step, or separately, presence of TSE infectivity is detected by detection of dimers of prion protein.



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Raven, Neil

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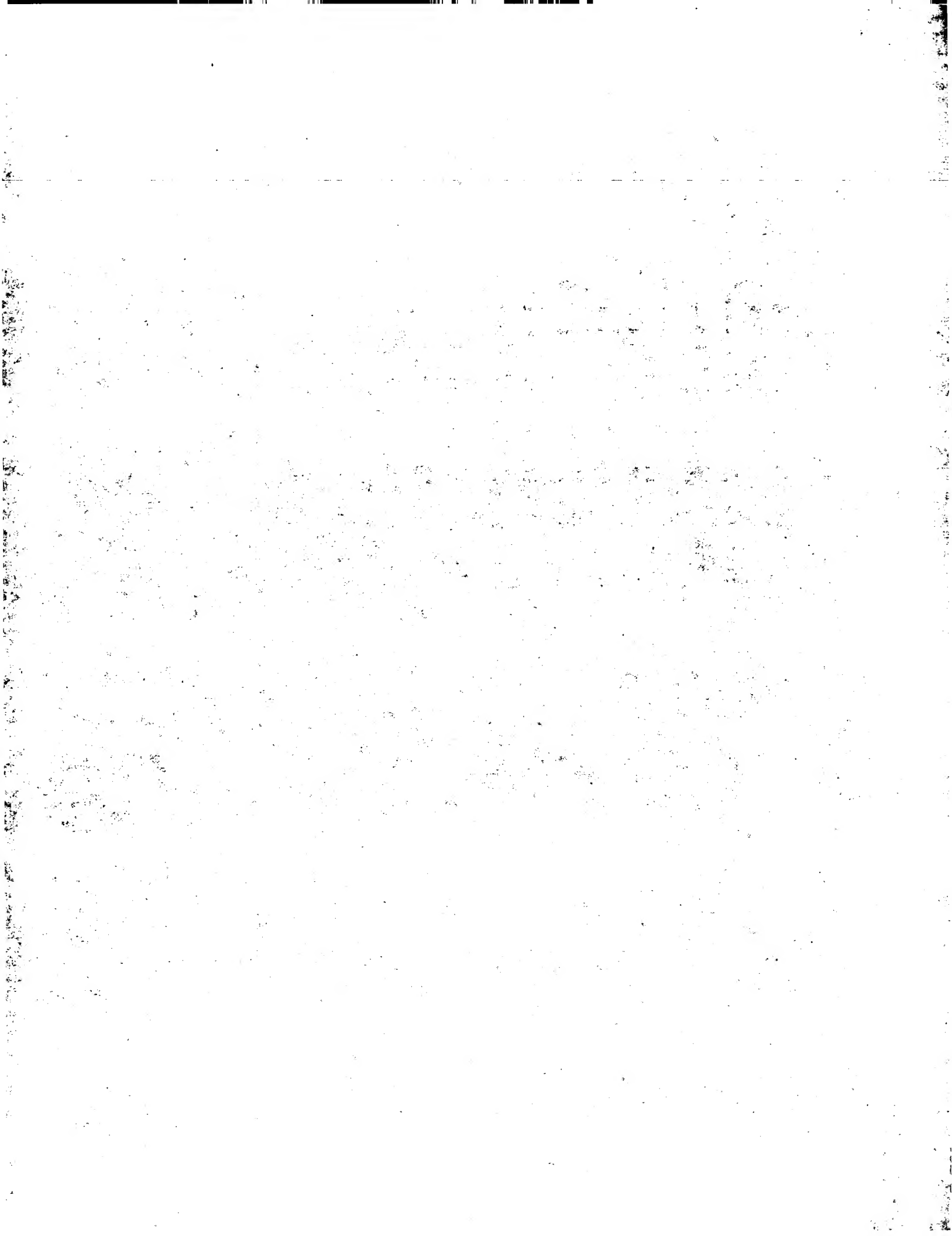
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 100 105 110  
 Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly  
 115 120 125  
 Pro Thr Gly Ser Thr Ala Leu Lys Thr Val Val Asp Lys Ala Val Ser  
 130 135 140  
 Ser Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly  
 145 150 155 160  
 Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala  
 165 170 175  
 Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala  
 180 185 190  
 Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr  
 195 200 205  
 Leu Pro Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr  
 210 215 220  
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Thr  
 225 230 235 240  
 Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr  
 245 250 255  
 Leu Gly Asn Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala  
 260 265 270  
 Ala Ala Gln  
 275

<210> SEQ ID NO 9  
 <211> LENGTH: 274  
 <212> TYPE: PRT  
 <213> ORGANISM: B. licheniformis

<400> SEQUENCE: 9

Ala Gln Thr Val Pro Tyr Gly Ile Pro Leu Ile Lys Ala Asp Lys Val  
 1 5 10 15  
 Gln Ala Gln Gly Phe Lys Gly Ala Asn Val Lys Val Ala Val Leu Asp  
 20 25 30  
 Thr Gly Ile Gln Ala Ser His Pro Asp Leu Asn Val Val Gly Gly Ala  
 35 40 45  
 Ser Phe Val Ala Gly Glu Ala Tyr Asn Thr Asp Gly Asn Gly His Gly  
 50 55 60  
 Thr His Val Ala Gly Thr Val Ala Ala Leu Asp Asn Thr Thr Gly Val  
 65 70 75 80  
 Leu Gly Val Ala Pro Ser Val Ser Leu Tyr Ala Val Lys Val Leu Asn  
 85 90 95  
 Ser Ser Gly Ser Gly Ser Tyr Ser Gly Ile Val Ser Gly Ile Glu Trp





-continued

100	105	110
Ala Thr Thr Asn Gly Met Asp Val Ile Asn Met Ser Leu Gly Gly Ala		
115	120	125
Ser Gly Ser Thr Ala Met Lys Gln Ala Val Asp Asn Ala Tyr Ala Arg		
130	135	140
Gly Val Val Val Val Ala Ala Gly Asn Ser Gly Asn Ser Gly Ser		
145	150	155
Thr Asn Thr Ile Gly Tyr Pro Ala Lys Tyr Asp Ser Val Ile Ala Val		
165	170	175
Gly Ala Val Asp Ser Asn Ser Asn Arg Ala Ser Phe Ser Ser Val Gly		
180	185	190
Ala Glu Leu Glu Val Met Ala Pro Gly Ala Gly Val Tyr Ser Thr Tyr		
195	200	205
Pro Thr Asn Thr Tyr Ala Thr Leu Asn Gly Thr Ser Met Ala Ser Pro		
210	215	220
His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Leu		
225	230	235
Ser Ala Ser Gln Val Arg Asn Arg Leu Ser Ser Thr Ala Thr Tyr Leu		
245	250	255
Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Glu Ala Ala		
260	265	270
Ala Gln		

<210> SEQ ID NO 10  
 <211> LENGTH: 269  
 <212> TYPE: PRT  
 <213> ORGANISM: B. lentus

<400> SEQUENCE: 10

Ala Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala		
1	5	10
His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp		
20	25	30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser		
35	40	45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr		
50	55	60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu		
65	70	75
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala		
85	90	95
Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala		
100	105	110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser		
115	120	125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly		
130	135	140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser		
145	150	155
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln		
165	170	175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile		
180	185	190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr		



-continued

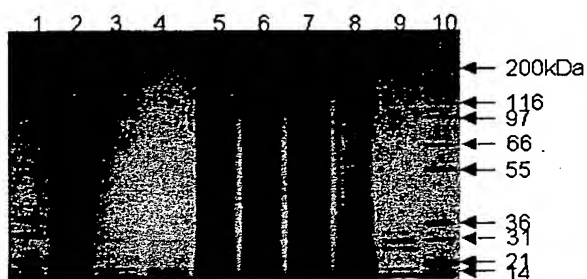
195	200	205
Ala Ser Leu Asn Gly Thr	Ser Met Ala Thr	Pro His Val Ala Gly Ala
210	215	220
Ala Ala Leu Val Lys Gln	Lys Asn Pro Ser Trp	Ser Asn Val Gln Ile
225	230	235 240
Arg Asn His Leu Lys Asn Thr	Ala Thr Ser Leu Gly Ser Thr	Asn Leu
245	250	255
Tyr Gly Ser Gly Leu Val Asn	Ala Glu Ala Ala Thr Arg	
260	265	



# **FIGURES 1-22**

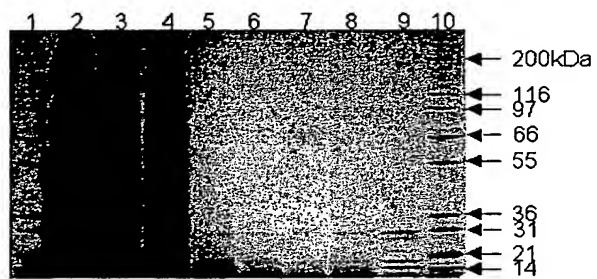


**Fig. 1**



Lanes 1 & 10, marker proteins; lane 2 untreated mbh; lane 3, 50°C; lane 4, 60°C; lane 5, 70°C; lane 6, 80°C; lane 7, 90°C; lane 8, 100°C; lane 9, Protease M.

**Fig. 2**

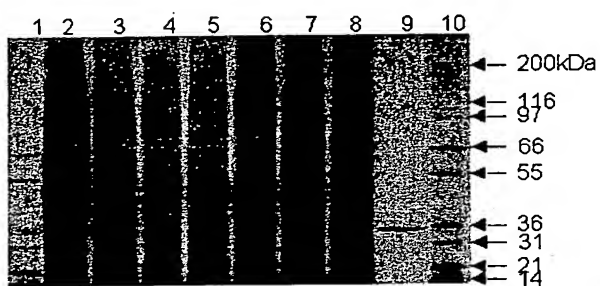


Lanes 1 & 10, marker proteins; lane 2 untreated mbh; lane 3, pH2; lane 4, pH4; lane 5, pH6; lane 6, pH8; lane 7, pH10; lane 8, pH12; lane 9, Protease M.



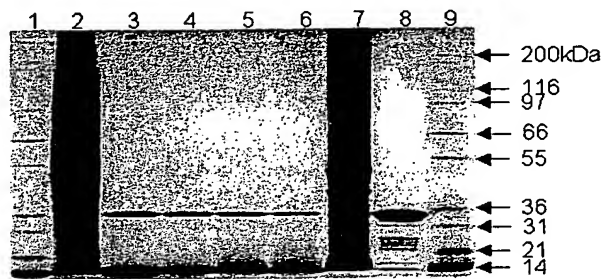


**Fig. 3**



Lanes 1 & 10, marker proteins;  
lane 2 untreated mbh; lanes 3 - 8,  
Rokko digest ( $20\text{mg.ml}^{-1}$  -  
 $0.1\text{mg.ml}^{-1}$ ), lane 9, Rokko  
( $1\text{mg.ml}^{-1}$ ).

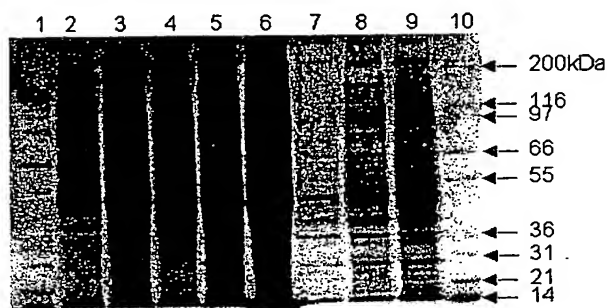
**Fig. 4**



Lanes 1 & 9, marker proteins; lane 2  
untreated mbh; lane 3, 2% SDS; lane  
4, 1% SDS; lane 5, 0.5% SDS; lane 6,  
0.25% SDS; lane 7, mbh + 2% SDS;  
lane 8, Rokko ( $20\text{mg.ml}^{-1}$ ).

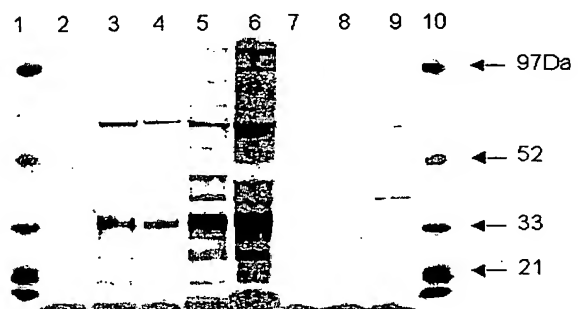


**Fig. 5**



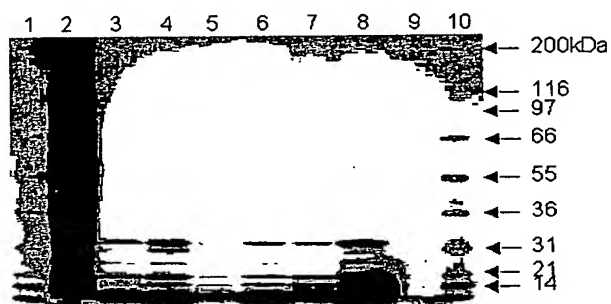
Lanes 1 & 10, marker proteins; lanes 2 & 3, mbh; lanes 4 - 6, mbh pellet; lanes 7 - 9, mbh supernatant.

**Fig. 6**



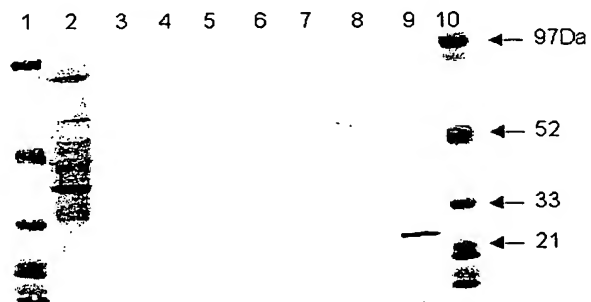


**Fig.7**



Lanes 1 & 10, marker proteins; lane 2, untreated mbh; lane 3, Protease G digest; lane 4, Protease G; lane 5, Protease R digest; lane 6, Protease R; lane 7, Protease C digest; lane 8, Protease C; lane 9, rec. mouse PrP.

**Fig.8**





1

2

3

4

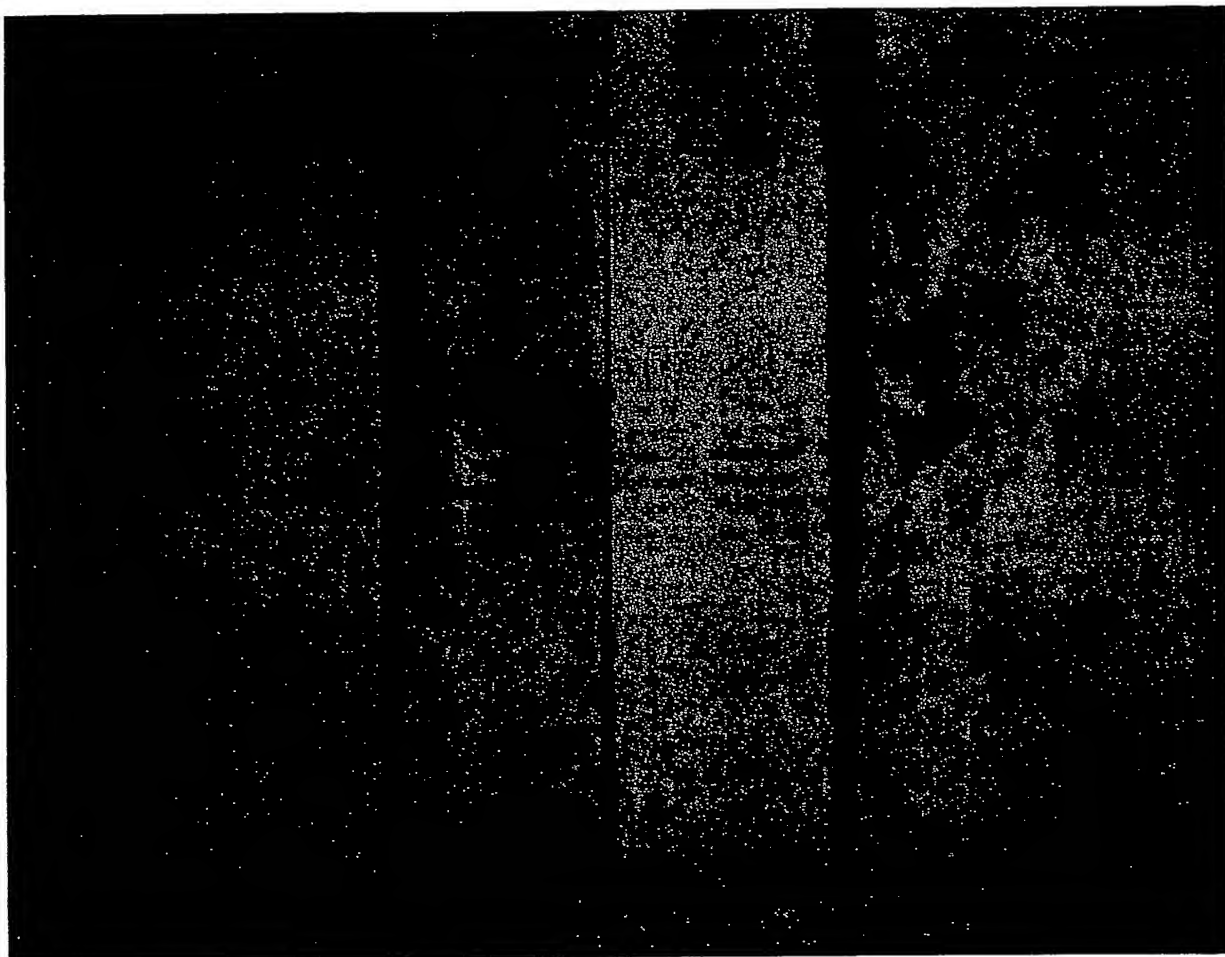


Fig. 9





5

6

7

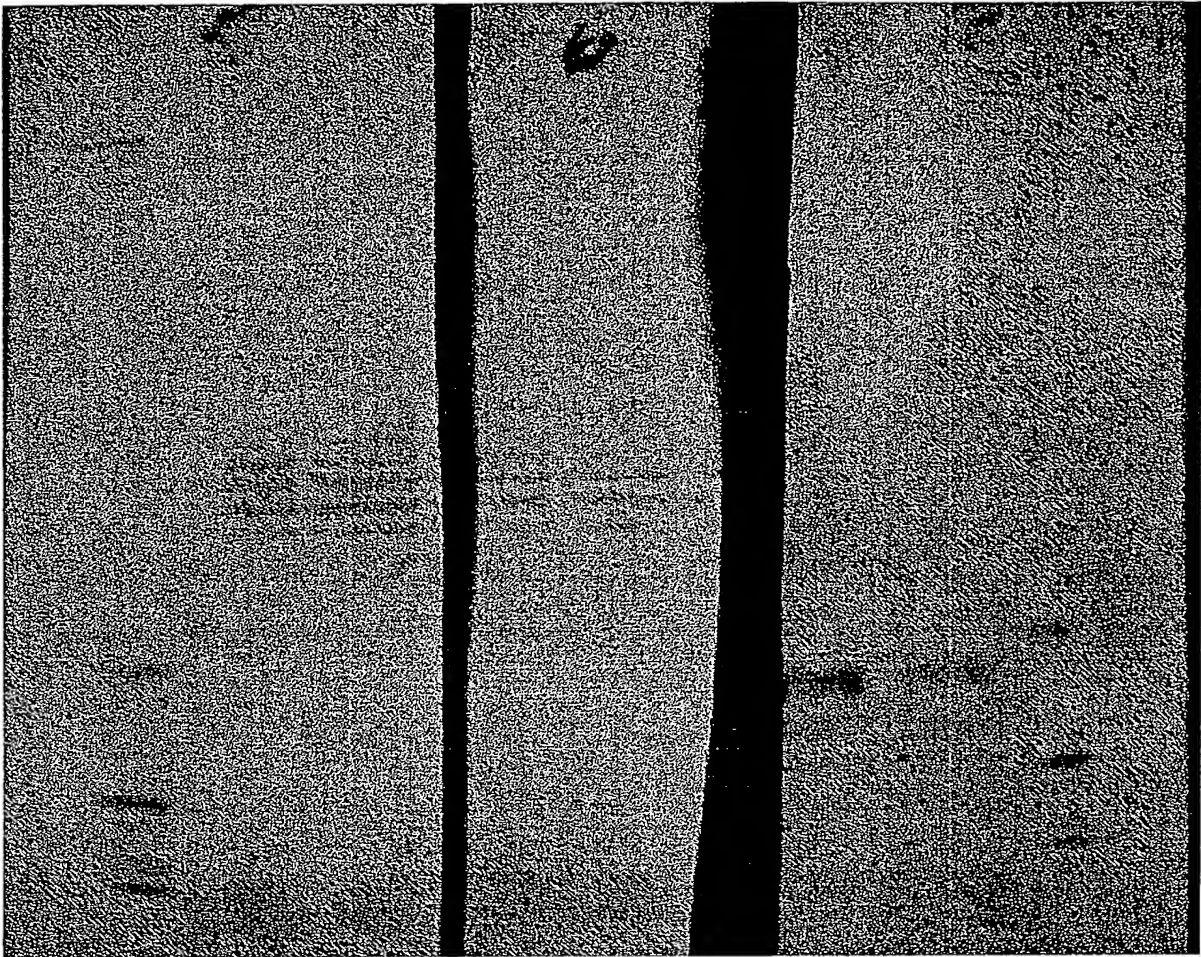


Fig. 10



1

2

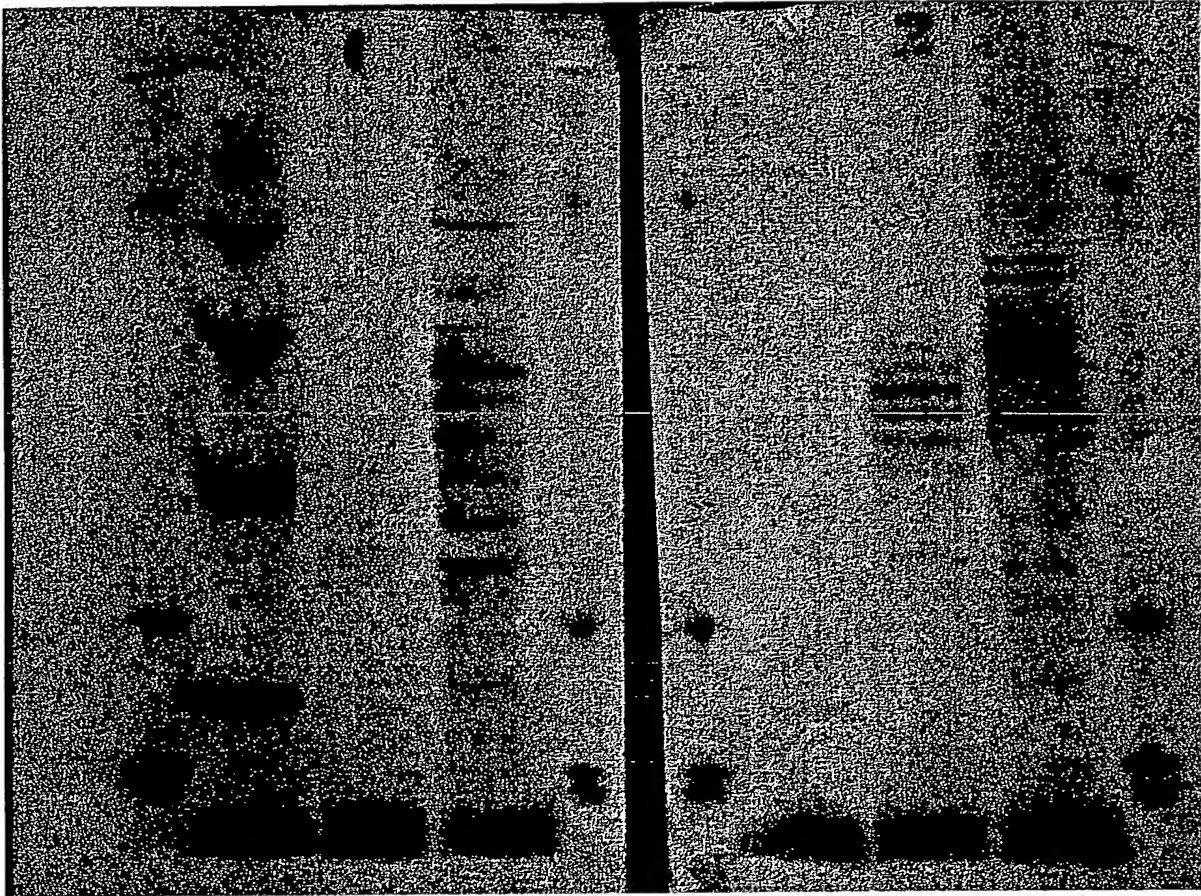


Fig. 11



3

7

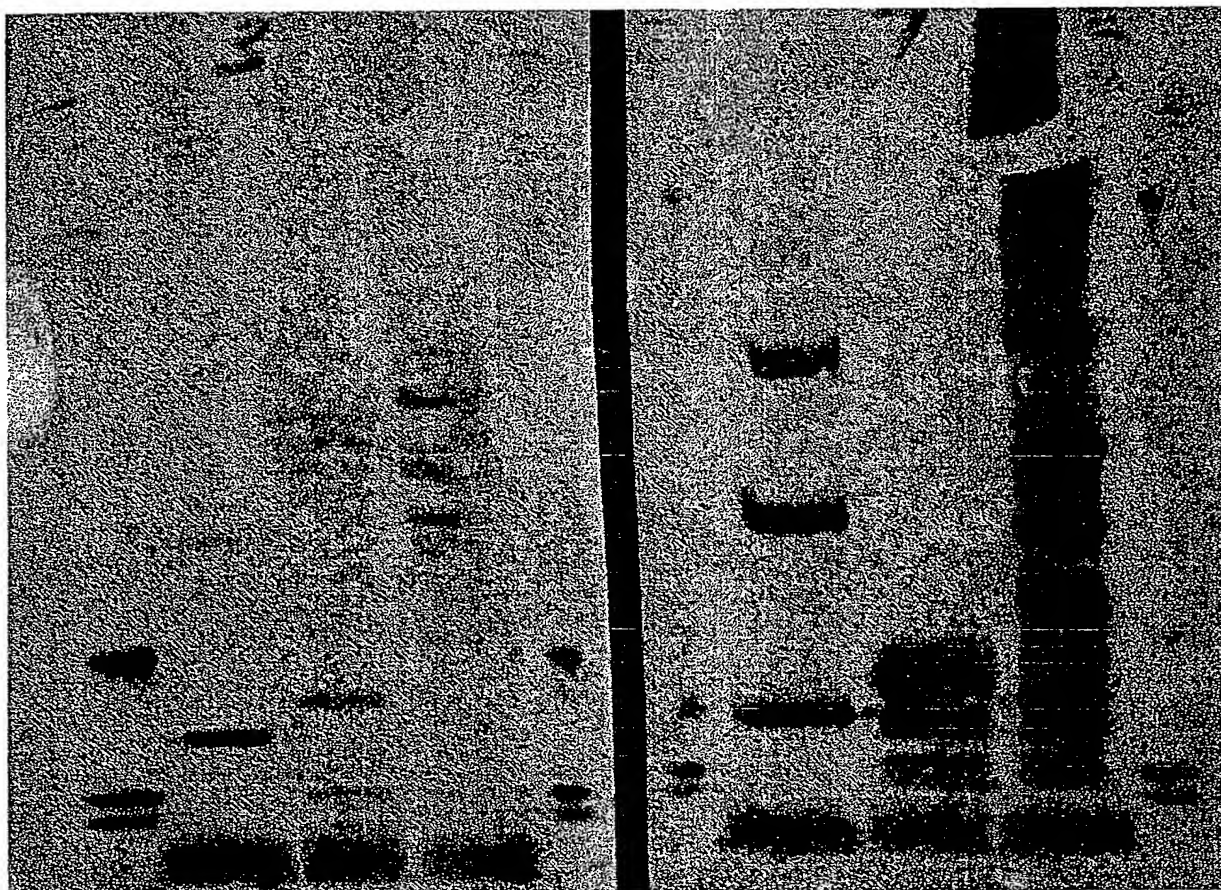


Fig. 12



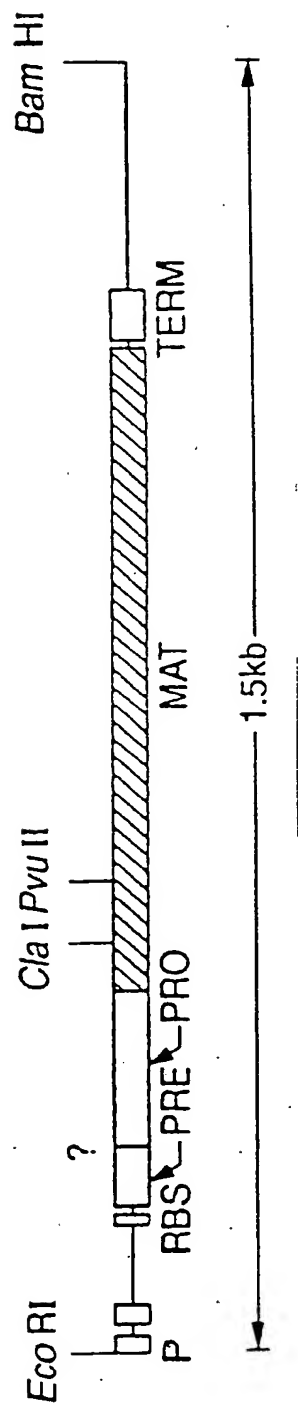


FIGURE 13.A





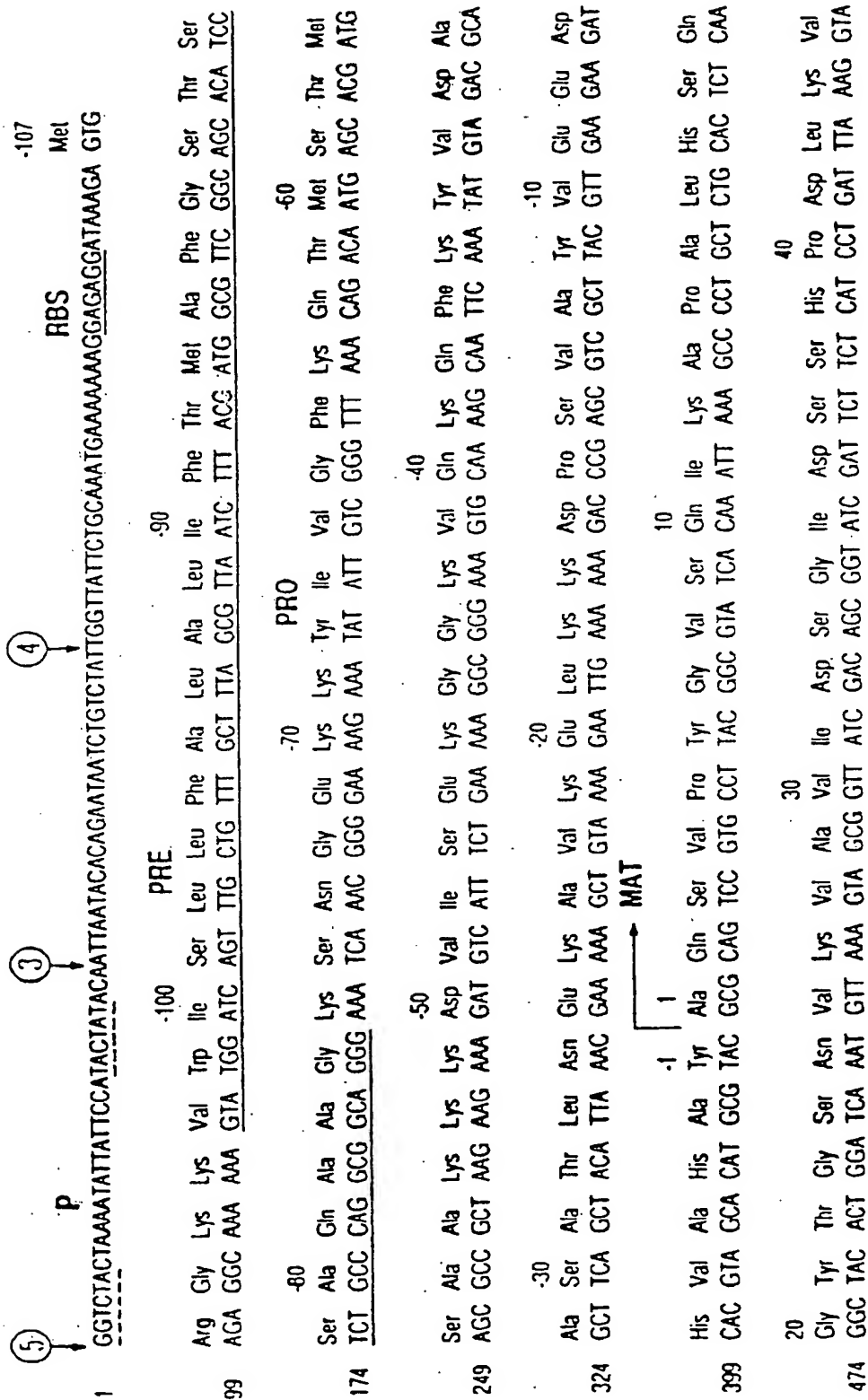


FIGURE 13.B1



549	Ala	Gly	Gly	Ala	Ser	Met	Val	Pro	Ser	Glu	Thr	Asn	Pro	Asn	60	Asp	Asn	Ser	His	Gly	Thr	His	Val	Ala	
	GCA	GGC	GGA	GCC	AGC	ATG	GTT	CCT	TCT	GAA	ACA	AAT	CCT	TTC	CNA	GAC	AAC	TCT	CAC	GGA	ACT	CAC	GTT	GCC	
70	Gly	Thr	Val	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu	Gly	Val	Ala	Pro	Ser	Ala	Ser	Leu	Tyr	Ala	Lys	
24	GGC	ACA	GTT	GCG	GCT	CTT	AAT	AAC	TCA	ATC	GGT	GTA	TTA	GGC	GTT	CCG	CCA	AGC	GCA	TCA	CTT	TAC	GCT	GTA	
699	Val	Leu	CTC	GGT	GCT	GAC	Gly	Ser	Gly	Gln	Tyr	Ser	Trp	Ile	110	Asn	Gly	Ile	Glu	Trp	Ala	Ile	Ala	Asn	Met
	GTT	CTC	GGT	GCT	GAC	GAC	GGT	TCC	GGC	CAA	TAG	AGC	TGG	ATC	ATT	AAC	GGA	ATC	GAG	TGG	CGC	ATC	GCA	AAC	
774	Asp	Ala	100	Val	Leu	Ile	130	Gly	Ser	Ala	Ala	Leu	Lys	Ala	Val	Asp	Lys	Ala	Val	Ala	GCC	GTT	GCA		
	GAC	GTT	ATT	AAC	ATG	AGC	CTC	GGC	GGA	CCT	TCT	GGT	TCT	GCT	GCT	TTA	AAA	GGC	GCA	GTT	GAT	AAA	GCC	GTT	
849	Ser	Gly	Val	Val	Val	Val	Ala	Ala	Ala	Gly	Ala	Gly	Asn	Glu	150	Gly	Thr	Ser	Ser	Thr	Val	Gly	Tyr	Pro	
	TCC	GGC	GTC	GTA	GTC	GTT	GCG	GCA	GCC	GGT	AAC	GAA	GAC	GGC	ACT	TCC	GGC	AGC	TCA	AGC	ACA	GTG	GGC	TAC	
924	Lys	Tyr	Pro	Ser	Val	Ile	Ala	Val	Gly	Ala	Val	Ala	Val	Val	180	Asp	Ser	Ser	Ser	Arg	Ala	Ser	Phe	Ser	
	AAA	TAC	CCT	TCT	GTC	ATT	GCA	GTA	GGC	GCT	GTT	GAC	AGC	AGC	MAC	CAA	AGA	GCA	TCT	TTC	TCA	AGC	GTA	GGA	
999	Glu	Leu	Asp	Val	Met	Ala	Pro	Gly	Val	Ser	Ile	Gln	Ser	Thr	Leu	Pro	Gly	Asn	Lys	Tyr	Gly	Ala	Tyr	Asn	
	GAG	CTT	GAT	GTC	ATG	GCA	CCT	GGC	GTA	TCT	ATC	CAA	AGC	ACG	CTT	CCT	GGA	AAC	AAA	TAC	GGG	GCG	TAC	AAC	
1074	Thr	Ser	Met	Ala	Ser	Pro	His	Val	Val	Ala	Gly	Ala	Ala	Ala	200	GGA	GCG	GCT	GCT	TTG	ATT	CTT	TCT	AAG	
	ACG	TCA	ATG	GCA	TCT	CCG	CAC	GTT	GCC	GCA	GCG	GCT	GCT	TTG	ATT	CTT	TCT	AAG	CAC	CCG	AAC	TGG	ACA	AAC	
															210	Gly	Ala	Tyr	Gly	Ala	Tyr	Asn	Gly		
															220	Thr	Ser	Met	Ala	Ser	Pro	His	Val	Val	
															230	Gly	Ala	Gly	Ala	Ala	Ala	Leu	Ile	Leu	
															240	Asn	Pro	His	Lys	Ser	Pro	Asn	Tyr	Thr	
																Asn	Thr	TGG	ACA	AAC	ACT				

FIGURE 13.B2



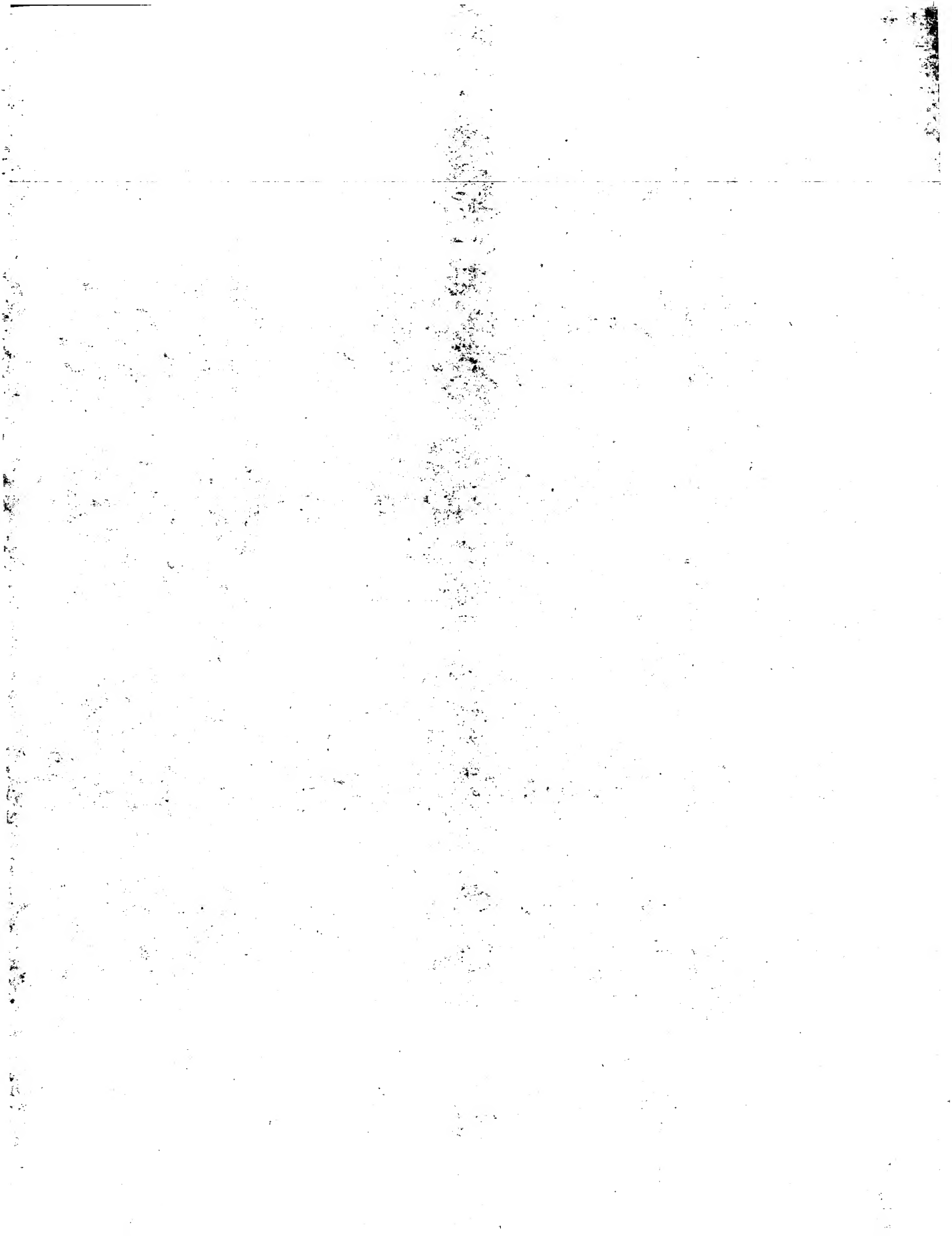




CONSERVED RESIDUES IN SUBTILISINS FROM  
*BACILLUS AMYLOLIQUEFACIENS*

1	10	20
A Q S V P . G . . . . .	A P A . H . .	G
21	30	40
. T G S . V K V A V . D . G . . . . .		H P
41	50	60
D L . . . G G A S . V P . . . . .		Q D
61	70	80
. N . H G T H V A G T . A A L N N S I G		
81	90	100
V L G V A P S A . L Y A V K V L G A . G		
101	110	120
S G . . S . L . . G . E W A . N . . . . .		
121	130	140
V . N . S L G . P S . S . . . . .		A . .
141	150	160
. . . . . G V . V V A A . G N . G . . . . .		
161	170	180
. . . . . Y P . . Y . . . . .		A V G A .
181	190	200
D . . N . . A S F S . . G . . L D . . . A		
201	210	220
P G V . . Q S T . P G . . Y . . . .		N G T
221	230	240
S M A . P H V A G A A A L . . . .		K . . .
241	250	260
W . . . Q . R . . L . N T . . . .		L G . .
261	270	
. . Y G . G L . N . . A A . . .		

FIGURE 14





COMPARISON OF SUBTILISIN SEQUENCES FROM:

*B. amyloliquefaciens*

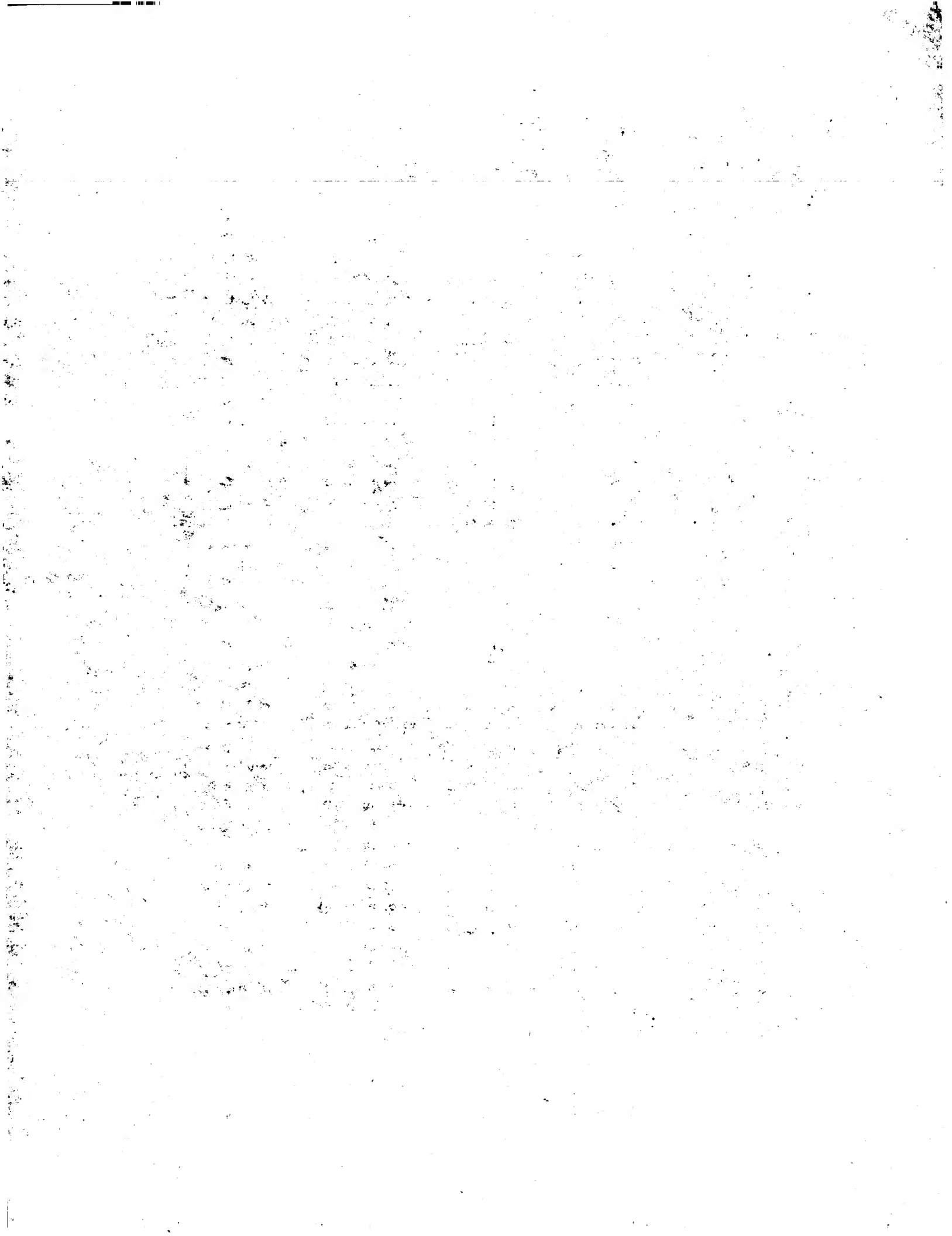
*B. subtilis*

*B. licheniformis*

*B. lentus*

01	10	20	30	
1 Q S V P Y G V S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P P				
A Q S V P Y G I S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P P				
A Q T V P Y G I P L I K A D K V Q A Q G F K G A N V K V A V L D T G I Q A S H P P				
A Q S V P W G I S R V Q A P A A H N R G L T G S G V K V A V L D T G I S T * H P P				
41	50	60	70	
D L K V A G G A S M V P S E T N P F Q D N N S H G T H V A G T V A A L N N S I G				
D L N V R G G A S F V P S E T N P Y Q D D G S S H G T H V A G T I A A L N N S I G				
D L N V V G G A S F V A G E A Y N * T D G N G H G T H V A G T V A A L D N T T G				
D L N I R G G A S F V P P G E * P S T Q D D G N G H G T H V A G T I A A L N N S I G				
81	90	100	110	
V L G V A P S A S L Y A V K V L G A D G S G Q Y S S W I I N G I E W A I A N N M D				
V L G V S P S A S L Y A V K V L D S T G S G Q Y S S W I I N G I E W A I S N N M D				
V L G V A P S V S L Y A V K V L N S S G S G S Y S S G I V S G I E W A T T N G M D				
V L G V A P S A E L Y A V K V L G A S G S G S V S S I A Q G L E W A G N N G M H				
121	130	140	150	
V I N M S L G G P S G S A A L K A A V D K A V A S G V V V V A A A G N E G T S G				
V I N M S L G G P T G S T A L K T V V D K A V S S G I V V A A A A A G N E G S S G				
V I N M S L G G A S G S T A M K Q A V D N A Y A R G V V V V A A A A G N S G N S G				
V A N L S L G S P S P S A T L E Q A V N S A T S R G V L V V A A S G N S G A G S				

Figure 15.A



161.	S	T	V	G	Y	P	P	G	K	Y	P	S	V	I	A	V	G	A	V	D	S	N	Q	R	A	S	F	S	S	V	G	P	E	L	D	V	M	A		
S	S	S	T	V	G	Y	P	P	A	K	Y	P	S	T	I	A	V	G	A	V	N	S	S	N	Q	R	A	S	F	S	S	A	C	S	E	L	D	V	M	A
S	T	S	T	V	G	Y	P	P	A	K	Y	D	S	V	I	A	V	G	A	V	D	S	S	N	R	A	S	F	S	S	V	G	A	E	L	E	V	M	A	
S	T	N	T	I	G	Y	P	P	A	K	Y	D	S	V	I	A	V	G	A	V	D	S	S	N	R	A	S	F	S	S	V	G	A	E	L	E	V	M	A	
* * *	*	I	S	Y	P	P	A	R	Y	A	N	A	M	A	V	G	A	V	G	A	T	D	Q	N	N	R	A	S	F	S	Q	Y	G	A	C	L	D	I	V	A

	201		210		229		230																																	
P	G	V	S	I	Q	S	T	L	P	G	N	K	Y	G	A	Y	N	G	T	S	M	A	S	P	H	V	A	G	A	A	L	I	L	S	K	H	P	N		
P	P	G	V	S	I	Q	S	T	L	P	G	N	K	Y	G	A	Y	N	G	T	S	M	A	S	P	H	V	A	G	A	A	L	I	L	S	K	H	P	T	
P	P	G	V	S	I	Q	S	T	L	P	G	N	K	Y	G	A	Y	N	G	T	S	M	A	S	P	H	V	A	G	A	A	L	I	L	S	K	H	P	N	
P	P	G	V	S	I	Q	S	T	L	P	G	N	K	Y	G	A	Y	N	G	T	S	M	A	S	P	H	V	A	G	A	A	L	I	L	S	K	H	P	N	
P	P	G	V	N	V	Q	S	T	Y	X	P	G	S	T	Y	A	S	L	N	G	T	S	M	A	T	P	H	V	A	G	A	A	L	V	K	Q	K	N	P	S

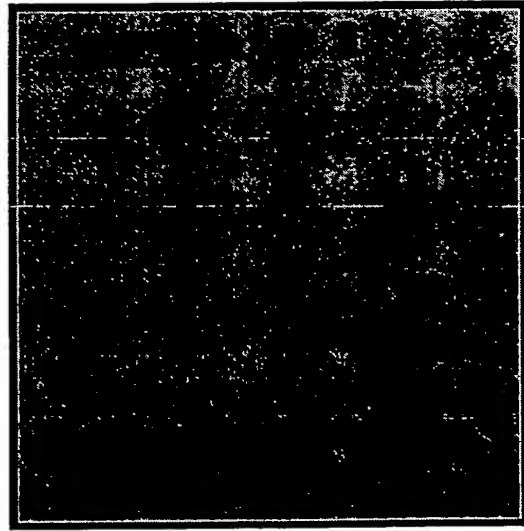
[illegible]

Figure 15.B



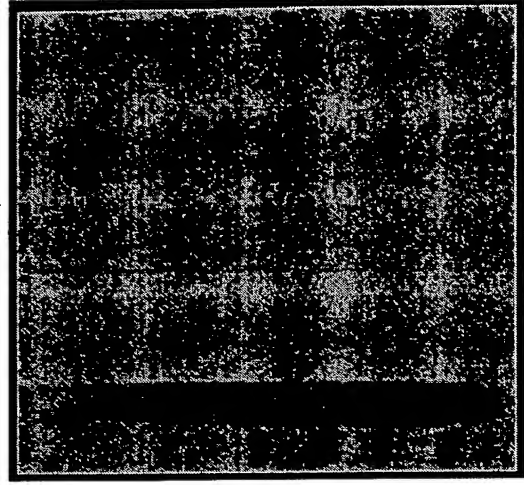
# Initial evaluation results

MC-A



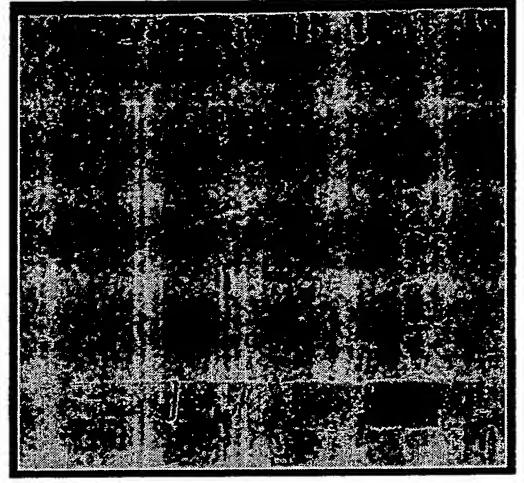
m mbh 2 4 6 8 10 12 P m

MC-3



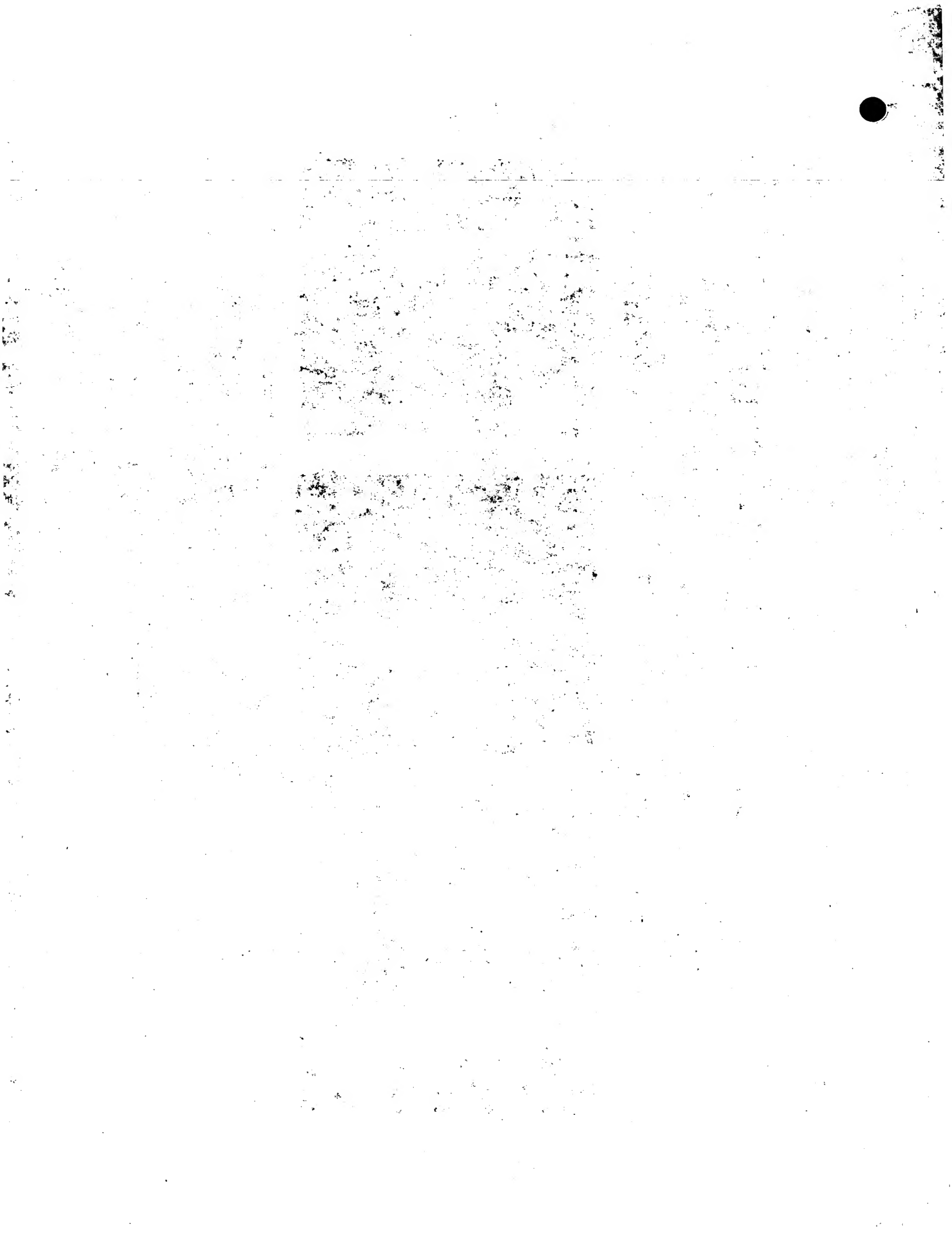
m mbh 2 4 6 8 10 12 P m

MC-4

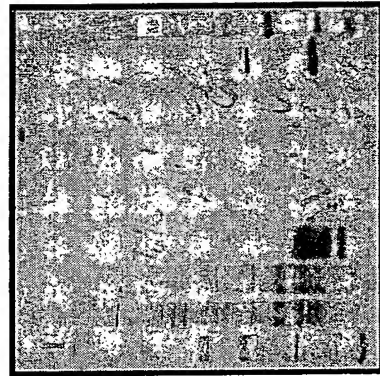


m mbh 2 4 6 8 10 12 P m

Fig. 16



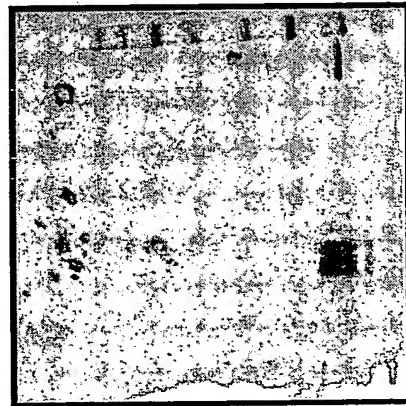
# Comparison with Properase



Properase 60°C 30 minutes

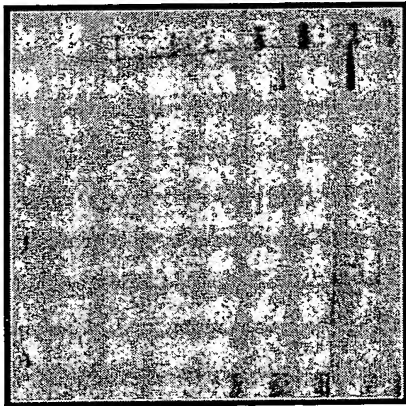
m 2 4 6 8 10 12 P rPrP m

MC-A 50°C 30 minutes



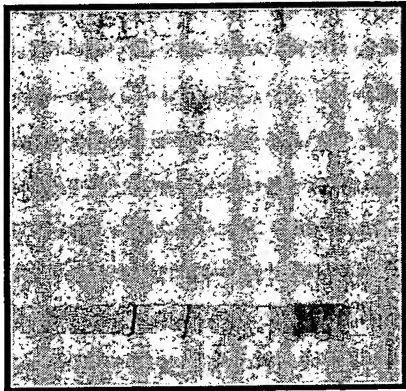
m 2 4 6 8 10 12 P rPrP m

MC-3 50°C 30 minutes



m 2 4 6 8 10 12 P rPrP m

MC-4 50°C 30 minutes



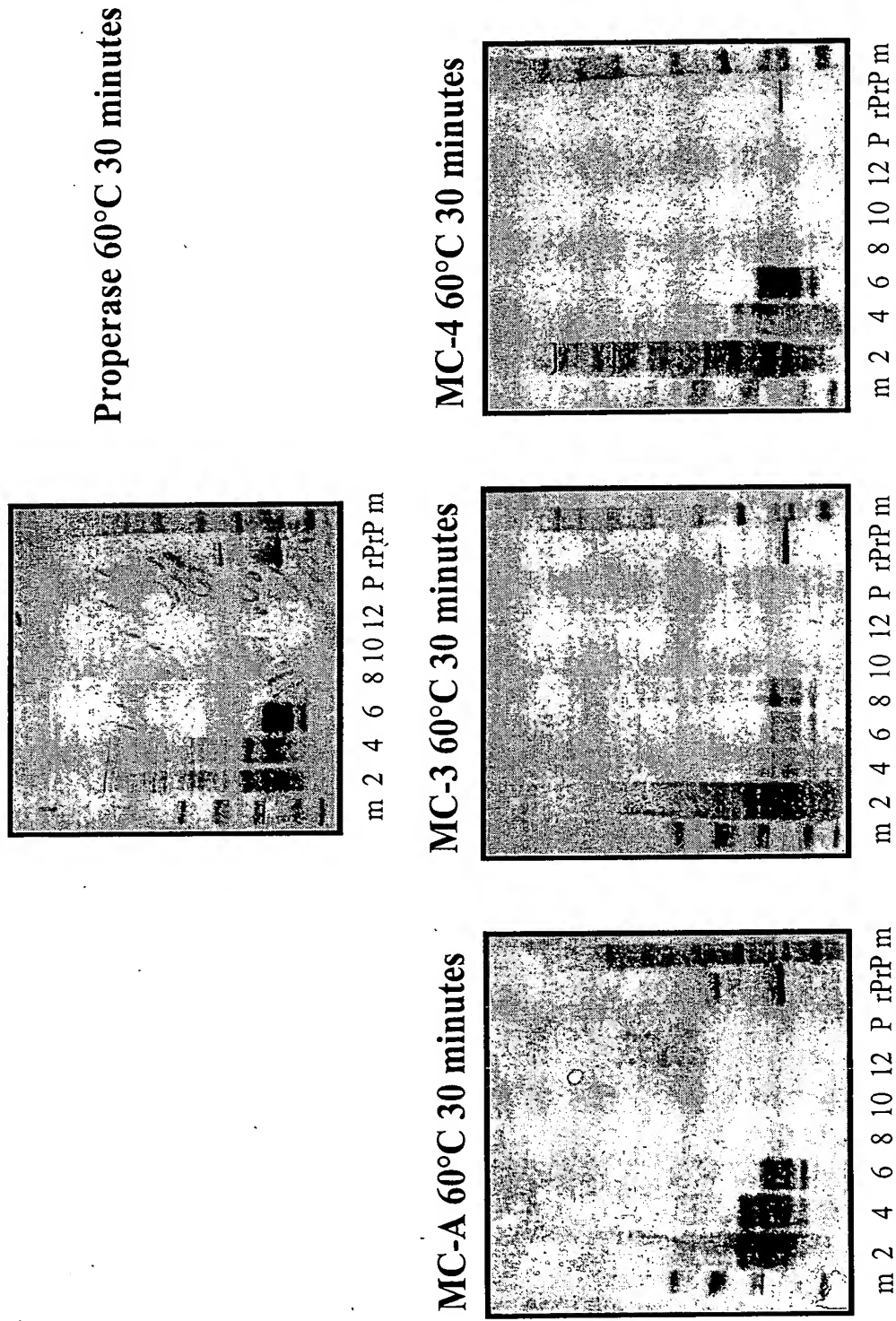
m mbh 2 4 6 8 10 12 P m

**Fig. 17**





# Comparison with Properase

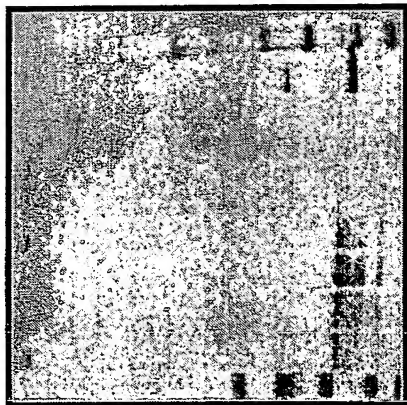


**Fig. 18**



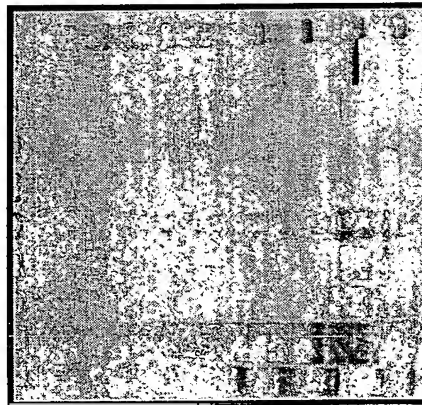
# Temperature profiling with MC-3

50°C 30 minutes



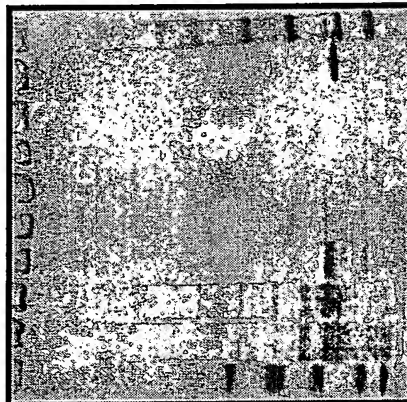
m 2 4 6 8 10 12 P rPrP m

60°C 30 minutes



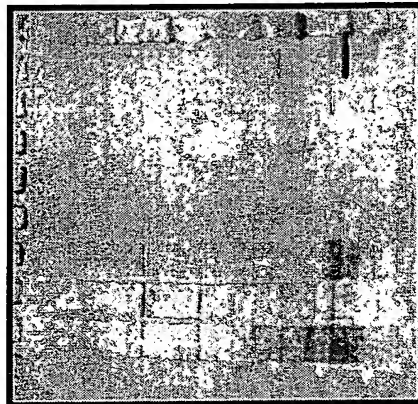
m 2 4 6 8 10 12 P rPrP m

70°C 30 minutes



m 2 4 6 8 10 12 P rPrP m

80°C 30 minutes



m 2 4 6 8 10 12 P rPrP m

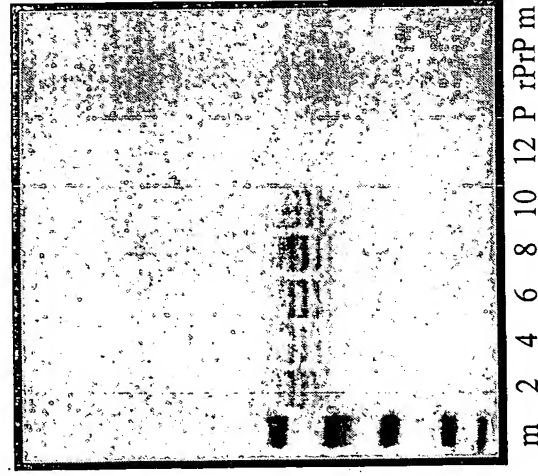
**Fig. 19**



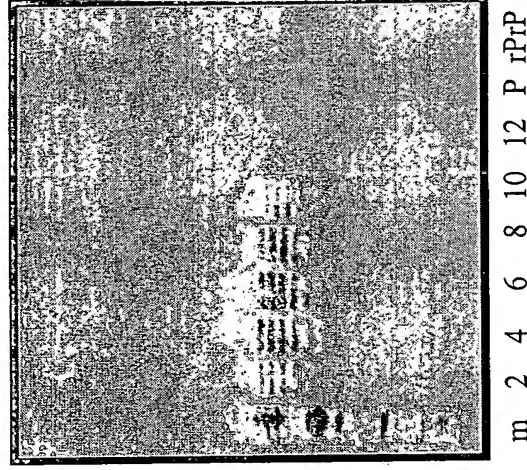
# Detection with PAb2

- mbh pH 2-12 digested at 50°C 30 minutes
- Detected with a chemiluminescent detection substrate (Pierce)

MC-A



MC-3



MC-4

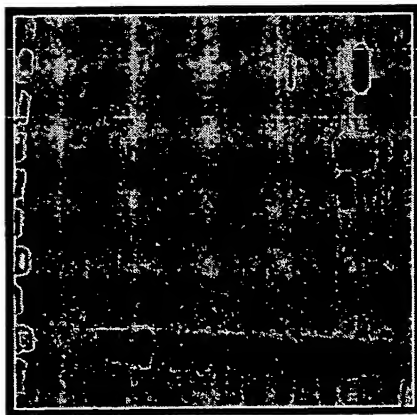


Fig. 20



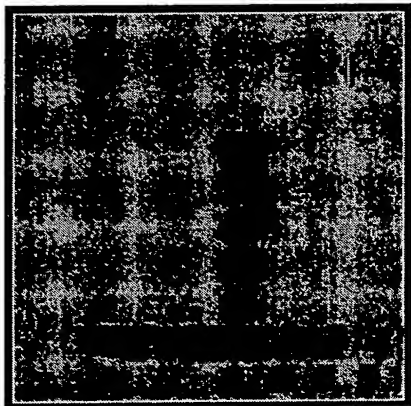
# MC-3 dilutions at pH 10 & pH 12

6H4 West Dura



m  
rPrP  
P  
1:100  
1:50  
1:20  
1:4  
n  
mbh  
m

PAb2 West Dura



m  
rPrP  
P  
1:100  
1:50  
1:20  
1:4  
n  
mbh  
m

pH 10

Monomer bands at  
1:20 dilution

HMW bands across  
dilution range

pH 12

No monomer bands

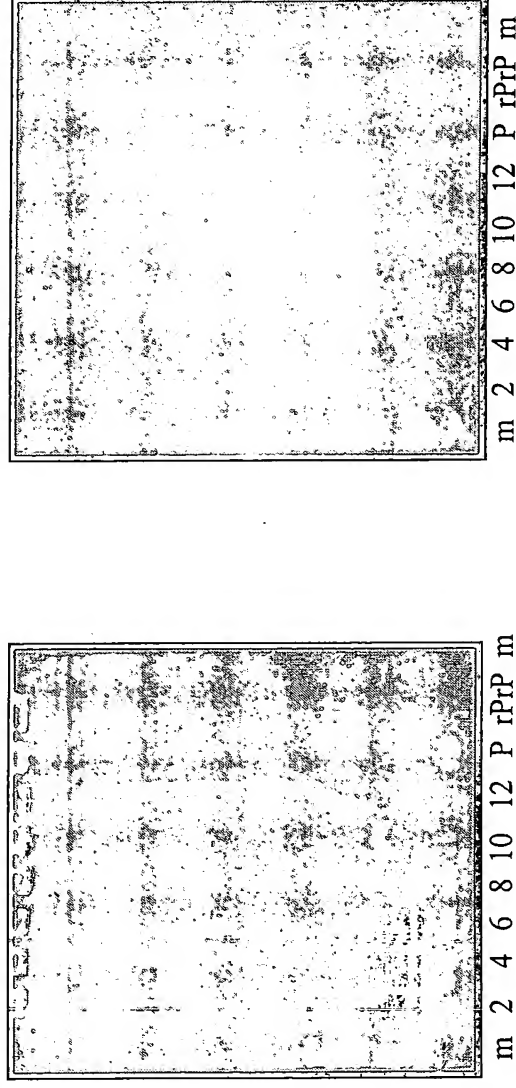
HMW bands much  
reduced across  
dilution range

Fig. 21





# Comparison with Proteinase K



- Characteristic PrP<sup>Sc</sup> monomer bands pH 2-10
- Incomplete digestion pH 12 however no clear monomers
- HMW bands present pH 2-12
- The new proteases are better at removing both the monomer and HMW bands than Proteinase K

Fig. 22

